BIOGENIC METHANE PRODUCTION FROM COAL USING METHANOGENIC MICROBIAL CONSORTIA

by

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ABSTRACT

Biogenic gas production is a promising alternative or supplement to conventional methane extraction from coalbeds. Microbial consortia native to coalbeds play an important role during biodegradation of carbonaceous sources to produce methane. It is possible to supplement and/or enhance the ability of indigenous microbial communities to produce methane from coal. Presume that adsorbed gas, generated over geologic time, can be supplemented with intentionally-generated biogenic gas during short-term engineering operations. There are two generic procedures for this. The first is to contact the coal with nutrients to support native bacterial development. The second concept is to inject appropriately cultured ex situ consortia. The research presented here involves this latter strategy.

Microbial populations were collected from various hydrocarbon-rich environments and locations characterized by biogenic methane production. Different rank coals, complex hydrocarbon sources, hydrocarbon seeps, and natural biogenic environments were incorporated in the sampling. Three levels of screening allowed selection of consortia, favorable nutrient amendments, and quantification of methane produced from various coal types. Incubation periods of up to twenty-four weeks were evaluated at 23°C. After a two-week incubation period, generated headspace gas concentrations reached 873,400 ppm (154 sft^3/ton) for methane and 176,370 ppm (31 sft^3/ton) for carbon dioxide. It was demonstrated that microbial communities from coal and lake sediments can be enriched

and adapted to effectively generate methane after initial atmospheric exposure.

Promising microbial consortia were subsequently incubated using low concentration of nutrient amendments (e.g., 22.4% v/v, 3.36 mg/cm^3 TSB) and [NaCl] 6.6 mg/cm^3 as a possible scenario and foresee the elevated costs of nutrient utilization at large-scale operations. Incubation periods of up to four months were evaluated at 23°C. After two months of incubation, generated headspace gas concentrations reached 95,700 ppm (14 sft^3/ton) for methane and 37,560 ppm (5.5 sft^3/ton) for carbon dioxide.

Finally, environmental conditions that led to increased methane production from subbituminous coal with a methanogenic consortium at low concentration of nutrient amendment were evaluated. A central composite design (CCD) was used to explore a broad range of operational conditions, examine the effects of the important environmental factors, such as temperature, pH, and salt concentration, and query a feasible region of operation to maximize methane production from coal. An anticipated detrimental effect of NaCl concentration on methane production was observed. The feasible region of operational conditions comprised pH values between 4.1 and 6.8, temperatures between 23°C and 37°C, and NaCl concentrations between 3.68 mg/cm^3 and 9.0 mg/cm^3. Coal biogasification was optimal at an initial pH value of 5.5, at 30°C, and a NaCl concentration 3.68 mg/cm^3 (i.e., 145,165 ppm, which is 25.6 sft^3/ton). Results infer that microbial consortia can be used as an attractive low-cost biological complements for coal biogasification.

This triumph is dedicated to: God for being my spiritual guide.
My parents, Gloria Córdoba R. and Roberto Fuertez Q. for being the people who have been permanently accompanying me during my life.
My brother Robert Fuertez C. for being my partner and permanent friend with whom I have shared all the moments of my life.

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1. LITERATURE REVIEW

1.1. Coal and Natural Gas Production

In the United States and many countries, including Mongolia, China, Russia, Australia, France, Canada, and India, there are challenges related to the anticipated shift from coal to natural gas (i.e., natural gas is mainly composed of methane: 90% - 99%). This fuel can be used for residential and commercial heating, electricity generation, transportation, and as an industrial feedstock. Additionally, methane offers many environmental benefits over other fossil fuels (Opara, 2012). According to the International Energy Outlook (2016), the consumption of natural gas worldwide is predicted to increase from 120 trillion cubic feet (Tcf) in 2012 to 203 Tcf by 2040. Natural gas consumption increases by 1.9%/year (IEO2016, p.9). To meet this growing demand, unconventional natural gas reservoirs (e.g., subsurface coalbeds, and shales) are being developed (Faiz and Hendry, 2006; Furmann, 2011; Green et al., 2008; Li et al., 2016; Meslé et al., 2013a, 2015c, 2015d; Park and Liang, 2016; Rathi et al., 2015; Ritter et al., 2015; Strapoc et al., 2011; Wray et al., 2009; Xiao et al., 2013).

Coalbed methane is found around the world, almost anywhere there is coal (Wray et al., 2009). The US. National Mining Association considers that 13% of the land mass of the United States, for instance, is underlain by coal deposits some of which contain commercial quantities of natural gas, commonly referred as coalbed methane (Furmann, 2011; Harris et al., 2008). Not all of this gas is safely and economically recoverable.

However, opportunities emerge for exploiting the carbon in the coal itself and generating supplementary volumes of natural gas.

1.2. Microbially Generated Coalbed Gas

Methane can have a thermogenic origin or due to biological activity. Thermogenic gas is formed by the chemical devolatilization of coal. This releases methane, which is typically a wet gas and generally found at depths greater than 1000 ft (304.8 m) in high rank coals. Biogenic methane, on the other hand, is the product of multiple biochemical reactions where coal is converted by the action of a complex group of indigenous microbes. Biogenic methane is typically a dry gas found at shallower in low rank coals (Alleman et al., 2005; Lavania et al., 2014; Strapoc et al., 2011).

Methane is already commercially produced or viable from coalbeds. Coalbed methane (CBM) production corresponds to an important fraction of natural gas reserves in the world. Up to 20% of natural gas produced from coal has been identified as microbial in origin (Ritter et al., 2015). Recognizing this, there is an opportunity for producing additional methane via stimulation by microorganisms. Biogenic coalbed gas has emerged as viable target for exploration and development and has currently received considerable research interest (Meslé et al., 2013a, 2013b). Research and development efforts have identified biogenic coalbed gas in more than 30 coal basins and about 35 sites worldwide (Flores, 2013). Biogenic gas is produced mostly mixed with thermogenic gas. The majority of coal basins in the United States and other countries share this characteristic. As an exception, the Powder River Basin in the US mostly contains biogenic gas (Flores, 2013; Strapoc et al., 2011).

Not only methane and carbon dioxide are present in CBM reservoirs. Nitrogen,

ethane, and small quantities of longer-chain hydrocarbons such as propane, butane, and pentane can be present. In addition, hydrogen sulfide, hydrogen, and argon have been encountered (Alleman et al., 2005; Clayton, 1997; Furmann, 2011; Seidle, 2011). Coalbed gas composition as well as indigenous microbial populations and metabolic pathways may vary between coal basins and within basins (Barnhart et al., 2013; Clayton, 1997; Rathi et al., 2015).

In recent years, various investigators have focused on enhancing biogenic methane production from different ranked coals (Fallgren et al., 2013; Green et al., 2008; Gupta and Gupta, 2014; Harris et al., 2008; Jin et al., 2010; Jones et al., 2010; Menger et al., 2000; Opara et al., 2012; Papendick et al., 2011; Pfeiffer et al., 2011; Rathi et al., 2015; Scott and Guyer, 2004; Ulrich and Bower, 2008; Wawrik et al., 2012; Zhang et al., 2015, 2016). Most published studies generally involve attempts to enhance microbial activity and methane production by adding nutrients. Conversely, microbial augmentation (i.e., inject appropriately cultured ex situ consortia) has received less attention (Park and Liang, 2016).

Emphasizing the adverse conditions that microbes might need to face during their implementation, important opportunities were identified for developing carbon-degrading microbial consortia, especially those that can tolerate atmospheric exposure and still retain anaerobic functionality. Methanogens are known as strict anaerobes (Wolfe, 2011), which makes it challenging to consider the application of strict anaerobic consortia without losing their activity during injection operations.

1.3. Pathways of Methane Generation

The biogenic formation of methane from complex organic matter (e.g., coal) requires at least three interacting metabolic groups (i.e., hydrolytic and fermentative

bacteria, acetogenic bacteria, and methanogens) participating in several steps (Boone, 1991). These microbes live symbiotically, relying on byproducts from one another. They are considered to be a bacterial consortium (Sparks, 2014).

Complex polymers such as polysaccharides, proteins, and lipids are initially degraded to oligomers and monomers such as sugars, amino acids, fatty acids, and glycerol with extracellular hydrolytic enzymes generated by primary fermentative bacteria (Figure 1). These oligomers and monomers are subsequent metabolized into fatty acids (e.g., lactate, succinate, and acetate), alcohols, H_2, and CO_2. Secondary fermenters or syntrophs convert higher molecular weight fermentation end-products (e.g., molecules with more than two carbon atoms) into acetate, H_2, CO_2, and possibly formate. Acetogenic bacteria as part of these secondary fermenters, oxidize the higher acids to acetate and H_2 or formate. Finally, methanogens or methane producers use the products of previous stage as substrates (Ali-Shah et al., 2014; Ferry, 1993; Megonigal et al., 2013; Meslé et al., 2013b). Methane, however, still can support additional anaerobic metabolism. Therefore, the actual final point of organic matter biodegradation could be the anaerobic oxidation of CH_4 to CO_2 (Megonigal et al., 2013; Valentine 2002).

It is accepted that methanogenesis proceeds using three general pathways (Meslé et al. 2013b). These are based on the source of carbon that is reduced to methane. They include:

- The reduction of CO_2 using H_2 as the electron donor via the hydrogenotrophic methanogenesis pathway (i.e., hydrogenotrophic methanogens are involved),
- One-carbon compounds (e.g., methanol, methylated amines) are reduced via the methylotrophic methanogenesis pathway (i.e., methylotrophic methanogens participate), and,

- Acetate is reduced via an acetoclastic methanogenesis pathway (i.e., characteristic of acetoclastic methanogens). In addition, acetate can be degraded into H_2 and CO_2 via syntrophic acetate oxidation. This may compete with acetogenesis (Ferry, 1993; Hedderich and Whitman, 2006; Meslé et al., 2013b).

Low energetic yield is attributed to methanogenesis. The most favorable and known reaction, which is conducted by most methanogens, is the reduction of CO_2 by H_2 with a $\Delta G^{\circ\prime}$ of -135 kJ/mol-CH_4 (Ferry, 1993; Meslé et al., 2013b). Conversion of CO_2 is the only methanogenic pathway having a net negative electron flow. Moreover, only a handful of electron donors, including hydrogen, formate, and alcohols, have been identified as suitable for this pathway. The lack of electrons and availability of the electron donors could be the reason why there is not more methane produced through this pathway (Opara, 2012).

Methylated compounds, on the other hand, can be simultaneously oxidized to CO_2, releasing six electrons, and reduced to methane through the reaction with coenzyme B, accepting two electrons. Lack of electron acceptors could be the limiting factor in this case (Opara, 2012). Alternatively, the free energy change ($\Delta G^{\circ\prime}$) is -31 kJ/mol-CH_4 for the acetoclastic reaction characteristic of genus Methanosarcina and Methanosaeta (Ferry, 1993; Meslé et al., 2013b). During the acetoclastic pathway two electrons are donated through the conversion of the carboxylic group into CO_2, while a series of reactions between the methyl group with coenzymes B, M, and tetrahydrosarcinapterin accepts two electrons, resulting in net zero-free electrons (Opara, 2012). Methyl-coenzyme M reductase (MCR), present in all known methanogens, catalyzes the final reduction of methane (Ferry, 1993; Meslé et al., 2013b).

Typical reactions carried out by methanogens during biodegradation of complex

organic matter and subsequent methanogenesis are shown in Table 1. Not all reactions occur for all methanogens. These are specific reactions for specific organisms (Ali-Shah et al., 2014; Ferry, 1993; Furmann, 2011; Opara, 2012; Zieminski and Frac, 2012). Even though there are only two genera identified as using the acetoclastic pathway, acetate reduction accounts for about two-thirds of methane production in freshwater and bioreactors (Ali-Shah et al., 2014; Opara, 2012).

Laboratory and field studies have indicated that methanogenesis may occur through different predominant mechanisms in different coalbeds and these mechanisms may even differ within a basin itself (Barnhart et al., 2013; Clayton, 1997; Rathi et al., 2015; Senthamaraikkannan, 2015; Senthamaraikkannan et al., 2016). For example, acetate has been found to be an important intermediate in coal seams in the Powder River Basin. This provided evidence to consider the acetoclastic methanogenesis as the dominant pathway.

However, other studies have showed that the hydrogenotrophic pathway may be dominant for the Powder River Basin (Senthamaraikkannan, 2015; Senthamaraikkannan et al., 2016). The proportional contribution of biogenic methane from the different pathways can depend on temperature, availability of nutrients, salinity, and presence of appropriate substrates (Faiz and Hendry, 2006).

1.4. Development of Methanogenic Microbial Consortia

Strict anaerobic conditions have been mostly used for the coal and in handling the microbial communities during experimentation (Furmann, 2011; Furmann et al., 2012; Green et al., 2008; Gupta and Gupta, 2014; Harris et al. 2008; Jones et al., 2010; Orem et al., 2010; Papendick et al., 2011; Rathi et al., 2015; Wawrik et al., 2012; Wolfe, 2011).

There has been relatively less research related to the development of aerotolerant methanogenic microbial consortia and their utilization. From a practical perspective, for large scale applications for both in situ and ex situ operations, these microbial communities should be seriously considered (Opara, 2012; Opara et al., 2012; Zhang et al., 2015).

Opara et al. (2012) conducted one of the first studies in this area, suggesting opportunities for further research and development. Zhang et al. (2015), for example, also considered the development of these microbial consortia. The key aspect of the bioaugmentation strategy is that aerotolerant microbial consortia can be injected in coal seams (where indigenous microbes are either unable or have limited capability to convert coal into methane). These consortia should tolerate oxygen exposure during their culturing, storage, and during injection into the target coalbed (in situ application). For ex situ applications, a microbial consortium with this characteristic can be used on coal waste heaps or bioreactors where oxygen exposure could be anticipated (Clement et al., 2012; Converse et al., 2001).

In the study conducted by Zhang et al. (2015), formation water was collected from a coalbed methane well in southern Illinois. Microbial populations were evaluated and a microbial consortium for ex situ bituminous coal bioconversion was developed. Ground coal was obtained from the Illinois basin. However, during their experimentation, initial air exposure was avoided. Nitrogen gas was used to purge the bioreactors prior to incubation of the microbes. Different media were used to culture microbes. After sixty-five days of incubation at 28°C, selected samples were added to fresh coal and nutrient medium. Some of these new samples were subsequently purged with nitrogen while others were initially in contact with the atmosphere. After twenty days of incubation, there was no statistically significant difference in the yields of methane and carbon dioxide at the

conditions tested. These researchers suggested that an effective gas-producing microbial consortium can be cultivated under conditions that are not strictly anaerobic.

Opara (2012) and Opara et al. (2012) evaluated methane production from various coal materials. Those authors developed aerotolerant microbial consortia that generated methane under conditions that are not strict anaerobic; coal and microbial inoculum were both exposed to air during sample collection, transfer, and handling. Bituminous coal and waste coal, lake sediments, wetland sediments, river sediments, digester sludge, as well as oil seep and gas well samples were used as sources for the microbial populations. An initial cultivation step with six different growth media was carried out after collection. Once microbes were cultured, gas production was evaluated using hydrocarbon materials (bituminous coal, waste bituminous coal, and lignite) with three levels of nutrient amendments (0%, 10%, and 50%, v/v-nutrient solution/ (nutrient and salt solution). This was done with a saline solution (8.5 mg/cm^3 NaCl), over a thirty-day period at 23°C. Based on this screening program, the best CO_2 and CH_4 producers were selected and combined into five final consortia; containing both methane and carbon dioxide generating microbes that were not sensitive to oxygen exposure during the entire experimental development.

As part of the research started by Opara et al. (2012) at the University of Utah and considering the possibilities of use of this novel technology for coal exploitation, a potential emerges for developing methanogenic microbial consortia. Thus, a methodology should be implemented to develop these consortia and assess their usability for coal biogasification.

1.5. Regulating Factors of Methane Production

Methanogenic biodegradation rates of coal can be influenced by the chemical composition. This is because different saturated hydrocarbons, aromatic hydrocarbons, resins, and asphaltenes fractions have different susceptibility to microbial degradation (Furmann, 2011; Furmann et al., 2012; Meslé et al., 2013b). With access to an appropriate substrate (e.g., coal), microbes can metabolize. This means building new cells (anabolism) and producing energy (catabolism) for their growth. In addition to a suitable substrate, the microbes require an adequate environment in order to thrive and function. Recognizing this, physicochemical factors such as temperature, salinity, and pH have been studied to determine their influence on microbial growth and methane production (Ali-Shah et al., 2014; Megonigal et al., 2013; Meslé et al., 2013b; Opara, 2012; Schnürer and Jarvis, 2010).

In a methane generation process where many different microorganisms participate, the environment has to be compatible with their requirements. This means that the environment may not be ideal for each microbe, but it still allows the organisms to grow and function (Schnürer and Jarvis, 2010). Microorganisms involved in a coal-degrading consortium have environmental requirements that can differ from those of methanogenic archaea. However, the requirements of methanogens are usually prioritized. Methanogens have longer regeneration time, slower growth and are more sensitive to environmental conditions than others organisms (Zupančič and Grilc, 2012). There is a diverse set of methanogenic populations that can have adaptations to function under specific conditions. However, an adverse environment with fluctuating or extreme conditions, as could be found in coals at depth, might reduce the possibility of significant natural biological methane production. Different environmental changes may have different consequences, generating possible shifts in microbial populations, which can be reflected in the overall

performance of the biologic system. Thus, a significant challenge emerges when a maximum biogenic methane production is sought in this scenario.

For bioaugmentation, the suitability of reservoir conditions for microbial communities should be taken into account since the environmental conditions influence methane production (Chuma et al., 2016; Head et al., 2014; Rathi et al., 2015). Therefore, the study of relevant factors (e.g., temperature, pH, salinity) and their influence on methanogenic communities is important for commercializing microbially-enhanced CBM generation.

1.5.1. The Effect of Oxygen

Since different microbial communities are present in a methane generating process, the importance of oxygen concentration varies considerably. Some microbes are very sensitive to oxygen exposure. Others can survive quite low oxygen concentrations, while others grow better if oxygen is in the surroundings. Microorganisms are usually categorized according to their relationship with oxygen. Strict anaerobes (i.e., only grow in absence of oxygen; they may die in the presence of oxygen and always perform anaerobic respiration or fermentation), and facultative anaerobes (i.e., they grow in the presence and absence of oxygen; respire with oxygen, but can switch to fermentation in the absence of oxygen) (Schnürer and Jarvis, 2010).

Methanogenesis is considered to be inhibited by oxygen. Methanogens are often considered to be strict (obligate) anaerobes that metabolize only in anaerobic environments and are extremely sensitive to this gas (Wolfe, 2011). Thus, strict anaerobic techniques are usually suggested for their study in laboratory settings (Furmann, 2011; Furmann et al., 2012; Wolfe, 2011). However, these microorganisms are not necessarily as oxygen

sensitive as has been thought. Some methanogens are fairly tolerant of O_2 and present certain adaptations that allow them to resist oxygen exposure for a period of time. It is plausible that some methanogens have evolved to handle different levels of oxygen. This hypothesis is based on the observation that their natural habitats are exposed to various oxygen levels for long periods of time (Botheju and Bakke, 2011; Kiener and Leisinger, 1983; Kirby et al., 1981; Megonigal et al., 2013).

Microbial aggregates such as flocs, granules, and biofilms may shield microbes living deep inside diffusion barriers and not allow the full penetration of oxygen. Steep oxygen gradients are created through those microbial aggregates due to the diffusion limitation and its possible consumption by facultative or aerobic microbes thriving closer to the surface of the aggregate or biofilm. Such a spatial organization of microbes may allow the interior methanogens and/or acetogens to survive in O_2-rich environments (Botheju and Bakke, 2011; Kato et al., 1997; Shen and Guiot, 1996).

Eventually, facultative, and/or aerobic microbes may consume this gas, creating favorable conditions for the development of obligate anaerobes. Thus, a temporary air leakage may not be a problem since these microorganisms can be able to rapidly consume the incoming oxygen (Ali-Shah et al., 2014; Opara 2012; Schnürer and Jarvis, 2010). However, there is no single lethal concentration of oxygen for a given anaerobe; a given concentration may or may not be lethal depending upon the age of the cell, its past history, and its present environment (Buswell and Neave, 1930).

Microorganisms responsible for fermentation or acidogenesis are composed of large quantities of facultative bacteria. These bacteria excrete enzymes to carry out the hydrolysis of complex organic matter. Although oxygen is considered to have negative effects, an improvement in the enzymatic hydrolysis has been obtained under certain

conditions (Botheju et al. 2009; Botheju and Bakke, 2011; Johansen and Bakke, 2006).

1.5.2. The Effect of Temperature

Temperature plays an important role in the performance of cells. An optimal growth temperature is characteristic for each culture. If this temperature is exceeded, the growth rate decreases and microbial activity can cease (Schnürer and Jarvis, 2010). Thus, a net decrease in concentration of viable cells can be observed. Generally, the optimum temperature (i.e., the temperature at which the organisms grow most rapidly and work most efficiently) is strongly linked to the environment from which the organism originates (Shuler and Kargi, 2002; Schnürer and Jarvis, 2010).

Microorganisms can be divided into different groups depending on the temperature at which they best thrive and grow. These groups include psychrophilic (around 10°C), mesophilic (around 35°C), thermophilic (above 50°C), extremophilic (above 65°C), and hyperthermophilic (above 85°C) (Schnürer and Jarvis, 2010). Among biological processes, methanogenesis is often more impacted by temperature (Megonigal et al., 2013). Many methanogenic microorganisms, especially mesophiles, have a preference for temperatures higher than 23°C (Megonigal et al., 2013; Opara, 2012; Ritter et al, 2015). Methanogenic microbes can grow in a variety of temperatures, ranging from marine sediments at ~2°C to geothermal areas above 100°C. There is a great diversity of mesophilic and thermophilic methanogens (Bergey and Holt, 1994; Ferry, 1993). However, the majority of known methanogens are mesophilic and grow optimally at temperatures between 30°C and 37°C (Ferry, 1993; Megonigal et al., 2013).

There is certain evidence that the dominant methanogenesis pathway can be influenced by temperature. However, contribution of methane production from acetoclastic

methanogenesis or hydrogenotrophic pathways also depends on nutrient availability, and the presence of appropriate substrates (Faiz and Hendry, 2006). Acetoclastic methanogens favor low to moderate temperatures and are found in young organic systems (i.e., fresh organic matter), while hydrogenotrophic methanogens related to carbon dioxide reduction may be predominant at high temperatures in older systems or deeper sediments (Faiz and Hendry, 2006; Senthamaraikkannan, 2015; Senthamaraikkannan et al., 2016).

For anaerobic degradation of organic matter (e.g., submerged rice field soil), incubation experiments, have shown changes in the methane production pathway as well as in the structure of the methanogenic community with temperature shifts. Methanogenic groups can be predominant at different temperatures (Lu et al., 2015). As different microorganisms are involved during bioconversion of organic matter, microbial populations may differ in their response to temperature (Schnürer and Jarvis, 2010).

Considering the in situ utilization of microbial consortia for enhanced coalbed methane, the temperature constraint on microbial activity still require further exploration. Generally, methanogens become inactive at temperatures above about 65°C. Higher temperatures essentially sterilize a reservoir (Seidle, 2011). At temperatures above 60°C, the activity of methane producers is reduced to a greater degree than that of fermentative organisms, which often results in the accumulation of fatty acids (Schnürer and Jarvis, 2010). However, there are thermophilic/extremophilic methanogens that can thrive at temperatures above 65°C or even 100°C. Thus, the development of microbial consortia that include microorganisms adapted to extreme conditions would be important elements for coal biogasification at greater depths (Rathi et al., 2015; Seidle, 2011).

1.5.3. The Effect of pH

As in all biochemical process, pH has an important effect on the bioconversion of complex organic matter (Del Real, 2007; Green et al., 2008). Biologic activity may be reduced when conditions are far from an optimum pH (Del Real, 2007). The pH level has a significant influence on methane production since methanogens are the most affected group within a microbial community (Del Real, 2007; Zupančič and Grilc, 2012). Since there are different organisms participating in this process, their pH requirements for optimal growth vary greatly. While fermenting, acid-producing microorganisms manage to live in acidic conditions, down to pH 5.0 (Schnürer and Jarvis, 2010), many methanogenic microbes have shown optimal growth conditions with pH values close to neutral (6.8 – 7.4) (Megonigal et al., 2013).

Most methanogenic communities seem to be dominated by neutrophilic species with limited growth and methane production when the pH is below 6 or above 11 (Gupta and Gupta, 2014; Megonigal et al., 2013; Opara, 2012). This indicates that some methanogens can remain active outside a pH-neutral range (Ferry, 1993). The tolerance of acid-forming organisms to lower pH is illustrated by the fact that decomposition of substrate often begins with acid formation and low pH as a result. Methane production does not usually take place at this condition because pH is too low (Schnürer and Jarvis, 2010).

However, there are known methanogens that can exist in extreme pH environments. Values of pH equal to 4 or even below are characteristic of some peat bogs where methane has been produced. Methanogenic activity has been reported when peat samples were incubated at pH 3.0. Methanogens of the genus *Methanobacterium* have been found to grow at pH values as low as 5 and to produce some methane down to pH 3 (Ferry, 1993). Maximum rates between pH 5.0 to 6.0 and temperatures up to 30°C have been observed

for the genus *Methanobacterium* (Kotsyurbenko et al., 2007). The dominance of hydrogenotrophic methanogenesis at pH 3.8 and temperatures of 4°C or 15°C has been reported. In addition, studies in bog sediments have showed that both CO_2 reduction and acetoclastic methanogenesis pathway could occur at the low pH value of 4 with an optimal condition at pH between 5 and 6 (Kotsyurbenko et al., 2007). Ali-Shah et al. (2014) reported that methanogens, which decompose acetates (e.g., *Methanosarcina barkeri* and *Methanosarcina sp*.), have been isolated from environments at pH 5, while methylotrophic and hydrogenotrophic methanogens have been found in strongly alkaline ecosystems. Moderately alkaliphilic methanogens that are able to grow optimally near pH 8 and 9 have also been found (Ferry, 1993; Goodwin and Zeikus, 1987; Kotsyurbenko et al., 2007; Williams and Crawford, 1985).

Kotsyurbenko et al. (2007) observed important effects of pH on the rate of methanogenesis, the methane production pathway and predominant methanogenic community. A shift from acetoclastic to H_2-dependent methanogenesis was found between pH 4.7 and 3.8 in an acidic peatland. This behavior was explained by the likely presence of acetic acid in its free form (not dissociated) at pH less than 4.7, inhibiting acetoclastic methanogens (Kotsyurbenko et al., 2007; Megonigal et al., 2013). However, this explanation may not be unique. Acetoclastic methanogens and other microorganisms, as was above mentioned, have also been found in environments with low pH and might have specialized mechanisms to compensate for decoupling by acetic acid (Kotsyurbenko et al., 2007).

Kim et al. (2004) studying the production of hydrogen through anaerobic digestion of glucose in a semicontinuous reactor at 35°C, reported methanogenic activity at pH 4.5 in presence of butyrate and acetate. Similar behavior was observed when these

investigators conducted a batch experiment; most of the acetate remained unchanged over the entire test period indicating that the methane was produced by hydrogenotrophic methanogens. Methanogenic activity is usually reported to be inhibited by acidic conditions below pH 5.0. However, as mentioned, some hydrogenotrophic methanogens may sustain their activity, while acetoclastic methanogens are inhibited. Thus, it has been postulated that hydrogen utilizing methanogens may be more tolerant to the acidic conditions than other methanogens.

1.5.4. The Effect of Salinity

Microorganisms required salts to function. Salts contain essential building blocks (i.e., basic elements used during formation of new cells), such as sodium, potassium, and chloride. These substances are generally available in many substrates and do not need to be added. However, salts can also have a preservative effect that inhibits microbial growth. High salt concentrations, may cause a cell to pump out water and lose both form and function (Liu and Boone, 1991; Schnürer and Jarvis, 2010).

Methanogenic populations can be found over a wide range of salinities, ranging from fresh water to hypersaline scenarios (Ferry, 1993). Freshwater methanogens generally need at least $2.3x10^{-2}$ mg/cm^3 of sodium for their growth and metabolic functions (Ferry, 1993; Megonigal, et al., 2013; Patel and Roth, 1977). Typically, methanogens are usually the microorganisms that are most affected by increasing salt concentrations (Patel and Roth, 1977; Schnürer and Jarvis, 2010). Salt concentrations around 1.5 mg/cm^3 have inhibited methanogenesis. However, salt concentrations up to 8 mg/cm^3 can be tolerated by some methanogens (Schnürer and Jarvis, 2010). A salinity gradient, for instance, appears to correlate with methane production in the Antrim shale pore water (Michigan

Basin, US). A variation from dilute water to a NaCl concentration greater than 5 M (298 mg/cm^3) at the center of the basin correlates the limited methane production found in the sampling (Waldron et al., 2007).

The salt concentration that leads to significant inhibition may vary depending on various factors. Among these factors (e.g., temperature, pH, DO), the substrate may be included (e.g., acetate, H_2/CO_2) (Schnürer and Jarvis, 2010). Methylotrophic methanogens that use methanol, or methylamine compounds to produce methane may obtain more energy (-78.7 to -191.1 kJ/mol-substrate) than those methanogens that use H_2/CO_2 (-34 kJ/mol-substrate) or acetate (-31 kJ/mol-substrate) as substrates. This likely allows these methanogens to grow at higher salt concentrations (Waldron et al., 2007).

Hydrogenotrophic and acetoclastic methanogens are commonly found in subsurface settings and are generally limited to lower salinity environments (Waldron et al., 2007). *Methanocalculus halotolerants* is the most halotolerant hydrogenotrophic methanogen. This methanogen can survive NaCl concentrations up to 120 mg/cm^3, and was isolated from an oil field brine (Head et al., 2014; Waldron et al., 2007). On the other hand, there are a few known extremely halophilic methanogens. These are methylotrophs belonging to the family *Methanosarcinaceae*. An abundance of methylated osmoprotectants (e.g., betaine, dimethylpropiothetin) is characteristic of methylotrophic methanogens in hypersaline environments. The most halophilic methanogens correspond to the genus *Methanohalophilus*. Some of these methanogens can grow in salt concentrations up to 3M (175.3 mg/cm^3) (Ferry, 1993).

Generally, reduction in salinity might be seen as a key step for promoting methanogenesis where high salinities predominates. Microorganisms, either native or exotic consortia, may prefer salt concentrations less than 3M for optimal growth and

performance (Ritter et al., 2015). In addition, substrates fostering methanogenesis at lower salinity may not be the same as the substrates used at higher salinity, which suggests the probable dominance of determinate microbial populations and specific metabolic pathways (Waldron et al., 2007).

1.5.5. The Effect of Pressure

Elevated pressure is characteristic of many methanogenic environments (e.g., sediments underlying the deep ocean basins), and methanogenic activity is documented to pressures of 75Mpa (740.2 atm), equivalent to 7000m water depth (Hoehler et al., 2010). Difficulties associated with conducting studies at high pressures have limited the understanding of the tolerance and adaptations of methanogens at such conditions. However, there is evidence of survivability and growth for a methanogenic archaeon, *Methanothermobacter wolfeii* in a wide range of pressures (1-1200 atm) and temperatures (45-65°C) (Sinha et al., 2015).

Because biochemical and metabolic reactions occur in aqueous solution, high pressures might have only minimal effects on methane production (Hoehler et al., 2010). However, pressures substantially greater than 100Mpa (987 atm) are expected to denature proteins. The most relevant effects likely involve the enhanced solubility of gaseous substrates and products (e.g., H_2, H_2S) (Hoehler et al., 2010). Further investigation is still needed to determine the influence of pressure on coal biogasification.

Table 1. Important methanogenic reactions. Adapted from Ali-Shah et al. (2014).

	Reaction	ΔG°'(kJ/mol-CH_4)
Hydrogen	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135
Acetate	$CH_3COOH \rightarrow CH_4 + CO_2$	-31
Formate	$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	-145
Methanol	$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O$	-105
	$CH_3OH + H_2 \rightarrow CH_4 + H_2O$	-113
Carbon monoxide	$4CO + 2H_2O \rightarrow CH_4 + 3CO_2$	-196
Trimethylamine	$4(CH_3)_3N + 6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_3$	-75.8
Dimethylamine	$2(CH_3)_2NH + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_3$	-74.8
Methylamine	$4(CH_3)NH_2 + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_3$	-76.7
Dimethyl sulfide	$2(CH_3)_2S + 2H_2O \rightarrow 3CH_4 + CO_2 + 2H_2S$	-52.1

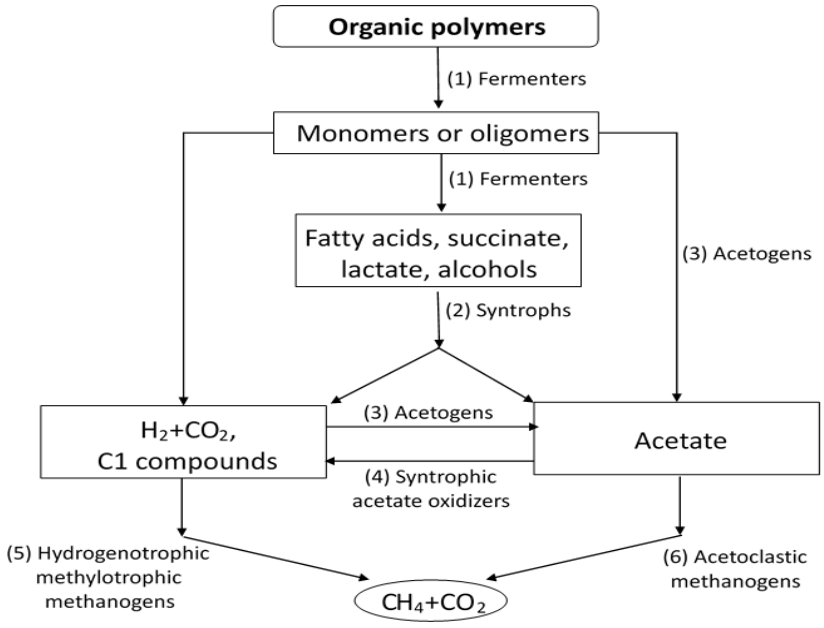


Figure 1. Schematic anaerobic food chain for the conversion of complex organic matter to methane. (1) Primary fermentative bacteria. (2) Secondary fermenters or syntrophs. (3) Acetogenic bacteria. (4) Syntrophic acetate oxidizers. (5) Hydrogenotrophic and methylotrophic methanogens. (6) Acetoclastic methanogens. Adapted from Meslé et al. (2013b).

1.6. References

Alleman, D., Duda, J.R., Lagiovane, P., 2005. Coalbed natural gas. U.S. Department of Energy. National Energy Technology Laboratory (NETL). https://www.netl.doe.gov/research/oil-and-gas (accessed 15.10.2016).

Ali-Shah, F., Mahmood, Q., Maroof-Shah, M., Pervez, A., Ahmad-Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Scientific World J. Art. No. 183752. http://doi.org/10.1155/2014/183752.

Barnhart, E.P., De León, K.B., Ramsay, B.D., Cunningham, A.B., Fields, M.W., 2013. Investigation of coal-associated bacterial and archaeal populations from a diffusive microbial sampler (DMS). Int. J. Coal Geol. 115, 64–70. https://doi.org/10.1016/j.coal.2013.03.006.

Bergey, D.H., Holt, J.G., 1994. Bergey's Manual of Determinative Bacteriology, ninth ed. Williams and Wilkins, Baltimore.

Botheju, D., Lie, B., Bakke. R., 2009. Oxygen effects in anaerobic digestion. Model Identif. Control. 30 (4), 191-201.

Botheju, D., Bakke. R., 2011. Oxygen effects in anaerobic digestion: A review. Open Waste Manag. J. 4, 1-19. doi:10.2174/1876400201104010001.

Buswell, E.G., Neave, S.L., 1930. Laboratory studies of sludge digestion. Illinois Division of State Water Survey, Bulletin No. 30. http://hdl.handle.net/2142/94553 (accessed 16.10.2016).

Chuma, O., Elebe, N., Gideon, O., 2016. Methanogen population of an oil production skimmer pit and the effects of environmental factors and substrate availability on methanogenesis and corrosion rates. Microb. Ecol. 72 (1), 175-184. doi:10.1007/s00248-016-0764-2.

Clayton, J., 1997. Geochemistry of coalbed gas: A review. Int. J. Coal Geol. 35 (1-4), 159-173. https://doi.org/10.1016/S0166-5162(97)00017-7.

Clement, B.G., Ferry, J.G., Underwood, S., 2012. Methods to stimulate biogenic methane production from hydrocarbon-bearing formations, U.S. Patent Application 20120138290 A1.

Converse, D., Hinton, S., Hieshima, G., Barnum, R., Mahankumar, S., 2001. Process for stimulating microbial activity in a hydrocarbon-bearing, subterranean formation, U.S. Patent Application 20010045279 A1.

Del Real, J.O., 2007. Evaluation and modeling of anaerobic depuration kinetics from stillage of alcohol industry. Ph.D. Dissertation. Institute of Basic Sciences and

Engineering, Autonomous University of Hidalgo State. Mexico.

Faiz, M., Hendry, P., 2006. Significance of microbial activity in Australian coal bed methane reservoirs – a review. Bull. Can. Pet. Geol. 54 (3), 261-272. doi:10.2113 /gscpgbull.54.3.261.

Fallgren, P., Jin, S., Zeng, C., Ren, Z., Lu, A., Ren, S., 2013. Comparison of coal rank for enhanced natural gas production. Int. J. Coal Geol. 115, 92-96. https://doi.org/ 10.1016/j.coal.2013.01.014.

Ferry J.G., 1993. Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall, New York.

Flores, R.M., 2013. Coal and Coalbed Gas: Fueling the Future, first ed. Elsevier Science, Burlington.

Furmann, A., 2011. Microbial methanogenesis from coal extracts in experimental bioreactors. M.Sc. Dissertation. Department of Geological Sciences, Indiana University. U.S.

Furmann, A., Schimmelmann, A., Brassell, S.C., Mastalerz, M., Picardal, F., 2013. Chemical compounds classes supporting microbial methanogenesis in coal. Chem. Geol. 339, 226-241. https://doi.org/10.1016/j.chemgeo.2012.08.010.

Goodwin, S., Zeikus, J., 1987. Ecophysiological adaptations of anaerobic bacteria to low pH: Analysis of anaerobic digestion in acidic bog sediments. Appl. Environ. Microbiol. 53 (1), 57-64.

Green, M.S., Flanegan, K.C., Gilcrease, P.C., 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76, 34-45. https://doi.org/10.1016/j.coal.2008.05.001.

Gupta, P., Gupta, A., 2014. Biogas production from coal via anaerobic fermentation. Fuel. 118, 238-242. https://doi.org/10.1016/j.fuel.2013.10.075.

Harris, S.H., Smith, R.L., Barker, Ch.E., 2008. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. Int. J. Coal Geol. 76, 46-51. https://doi.org/10.1016/j.coal.2008.05.019.

Head, I.M., Gray, N.D., Larter, S.R., 2014. Life in the slow lane: Biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front. Microbiol. 5, 1-23. doi:10.3389/fmicb.2014.0056 6.

Hedderich, R., Whitman, W., 2006. Physiology and biochemistry of the methane producing archaea, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt,

E. (Eds.), The Prokaryotes, third ed. Springer-Verlag., New York, pp. 1050-1079.

Hoehler, T., Gunsalus, R.P., Mcinerney, J.M., 2010. Environmental constraints that limit methanogenesis, in: Timmis, K.N., McGenity, T.J., Van der Meer, J.R., De Lorenzo, V. (Eds.), Handbook of Hydrocarbon and Lipid Microbiology. Springer Berlin Heidelberg., Berlin, pp. 635-654. doi:10.1007/978-3-540-77587-4_51.

International Energy Outlook, 2016. Chapter 3: Natural gas. DOE/EIA-0484. https://www.eia.gov/outlooks/ieo/ (accessed 18.11.2016).

Jin, S., Bland, A., Price, H., 2010. Biogenic methane production enhancement systems, U.S. Patent Application 7832475 B2.

Johansen, J.E., Bakke, R., 2006. Enhancing hydrolysis with microaeration. Water Sci. Technol. 53 (8), 43-50. doi:10.2166/wst.2006.234.

Jones, E., Voytek, M., Corum, M., Orem W., 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76, 7013-7022. doi:10.1128/AEM.00728-10.

Kato, M.T., Field, J.A., Lettinga, G., 1997. Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. Braz. J. Chem. Eng. 14 (4), 1678-4383. http://dx.doi.org/10.1590/S0104-66321997000400015.

Kiener, A., Leisinger, T., 1983. Oxygen sensitivity of methanogenic bacteria. Syst. App. Microbiol. 4 (3), 305-312. https://doi.org/10.1016/S0723-2020(83)80017-4.

Kim, In. S., Hwang, M.H., Jang, N.J., Hyun, S.H., Lee, S.T., 2004. Effect of low pH on the activity of hydrogen utilizing methanogen in bio-hydrogen process. Int. J. Hydrogen Energy. 29 (11), 1133-1140. https://doi.org/10.1016/j.ijhydene.2003.08.017.

Kirby, T., Lancaster, J., Fridovich, I., 1981. Isolation and characterization of the ion-containing superoxide dismutase of *Methanobacterium bryantii*. Arch. Biochem. Biophys. 210, 140-148.

Kotsyurbenko, O.R., Friedrich, M.W., Simankova, M.V., Nozhevnikova, A.N., 2007. Shift from acetoclastic to H_2-dependent methanogenesis in a West Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacerium* strain. Appl. Environ. Microbiol. 73 (7), 2344-2348. doi:10.1128/AEM.02413-06.

Lavania, M., Cheema, S., Manab, P., Ganapathi, R., 2014. Methanogenic potential of a thermophilic consortium enriched from coal mine. Int. Biodeterior. Biodegradation. 93, 177-185. https://doi.org/10.1016/j.ibiod.2014.05.022.

Li, X., Fu, X., Liu, A., An, H., Wang, G., Yang, X., Wang, L., Wang, H., 2016. Methane adsorption characteristics and adsorbed gas content of low-rank coal in China. Energy Fuels. 30 (5), 3840-3848. doi:10.1021/acs.energyfuels.6b00071.

Liu, Y., Boone, D.R., 1991. Effects of salinity on methanogenic decomposition. Bioresour. Technol. 35 (3), 271-273. https://doi.org/10.1016/0960-8524(91)90124-3.

Megonigal, J.P., Hines, M.E., Visscher, P.T., 2013. Anaerobic metabolism: Linkages to trace gases and aerobic processes, in: Turekian, K., Holland, H. (Eds.), Treatise on Geochemistry, second ed. Elsevier Inc., Oxford, pp. 273-359. doi:10.1016/B978-0-08-095975-7.00808-1.

Menger, W.M., Kern, E.E., Karkalits, O., Wise, D.L., Leuschner, A.P., Odelson, D., 2000. Microbial process for producing methane form coal, U.S. Patent Application. 6143534A.

Meslé, M., Periot, C., Dromart, G., Oger, P., 2013a. Biostimulation to identify microbial communities involved in methane generation in shallow, kerogen-rich shales. J. Appl. Microbiol. 114 (1), 55-70. doi:10.1111/jam.12015.

Meslé, M., Dromart, G., Oger, P., 2013b. Microbial methanogenesis in subsurface oil and coal. Res. Microbiol. 164 (9), 959-972. https://doi.org/10.1016/j.resmic.2013.07.004.

Meslé, M., Dromart, G., Haeseler, F., Oger, P., 2015c. Classes of organic molecules targeted by a methanogenic microbial consortium grown on sedimentary rocks of various maturities. Front. Microbiol. 6, 1-15. https://doi.org/10.3389/fmicb.2015.00589.

Meslé, M., Periot, C., Dromart, G., Oger, P., 2015d. Methanogenic microbial community of the eastern Paris basin: Potential for energy production from organic-rich shales. Int. J. Coal Geol. 149, 67-76. https://doi.org/10.1016/j.coal.2015.07.002.

Opara, A., Adams, D.J., Free, M.L., Mclennan, J., Hamilton, J., 2012. Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste materials. Int. J. Coal Geol. 96-97, 1-8. https://doi.org/10.1016/j.coal.2012.02.010.

Opara, A., 2012. Biochemically enhanced methane production from coal. Ph.D. Dissertation. Department of Civil and Environmental Engineering, University of Utah. U.S.

Orem, W.H., Voytek, M.A., Jones, E.J., Lerch, H.E., Bates, A.L., Corum, M.D., 2010. Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. Org. Geochem. 41 (9), 997-1000. https://doi.org/10.1016/j.orggeochem.2010.03.005.

Papendick, S.L., Downs, K.R., Vo, K.D., Hamilton, S.K., Dawson, G.K., Golding, S.D., 2011. Biogenic methane potential for Surat Basin, Queensland coal seams. Int. J. Coal Geol. 88, 123-134. https://doi.org/10.1016/j.coal.2011.09.005.

Park, Y.S., Liang, Y., 2016. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). Fuel. 166, 258-267. https://doi.org/10.1016/j.fuel.2015.10.121.

Patel, G.B., Roth, L.A., 1977. Effect of sodium chloride on growth and methane production of methanogens. Can. J. Microbiol. 23 (7), 893-897. doi:10.1139/m77-131.

Pfeiffer, R.S., Ulrich, G.A., DeBruyn, R.P., Vanzin, G., 2011. Chemical amendments for the stimulation of biogenic gas generation in deposits of carbonaceous material, U.S. Patent Application 7977282 B2.

Rathi, R., Priya, A., Vohra, M., Lavania, M., Lal, B., Sarma, P., 2015. Development of a microbial process for methane generation from bituminous coal at thermophilic conditions. Int. J. Coal Geol. 147-148, 25-34. https://doi.org/10.1016/j.coal.2015.06.004.

Ritter, D., Vinson, D., Barnhart, E., Akob, D., Fields, M., Cunningham, A., Orem, W., McIntosh, J., 2015. Enhanced microbial coalbed methane generation: A review of research, commercial activity, and remaining challenges. Int. J. Coal Geol. 146, 28-41. https://doi.org/10.1016/j.coal.2015.04.013.

Schnürer, A., Jarvis, A., 2010. Microbiological handbook for biogas plants. Swedish Waste Management U2009:03. Swedish Gas Centre Report 207. http://www.eac-quality.net/fileadmin/eac_quality/user_documents/3_pdf/Microbiological_handbook_for_biogas_plants.pdf (accessed 25.2.2015).

Scott, A.R., Guyer, J.E., 2004. Method of generating and recovering gas from subsurface formations of coal, carbonaceous shale and organic-rich shales, U.S. Patent Application 20040033557 A1.

Senthamaraikkannan, G., 2015. Development of multiscale microbial kinetic-transport models for prediction and optimization of biogenic coalbed methane production. Ph.D. Dissertation. Department of Chemical and Materials Engineering, University of Alberta. Canada.

Senthamaraikkannan, G., Budwill, K., Gates, I., Mitra, S.K., Prasa, V., 2016. Kinetic modeling of the biogenic production of coalbed methane. Energy Fuels. 30 (2), 871-883. doi:10.1021/acs.energyfuels.5b02450.

Seidle, J., 2011. Fundamentals of Coalbed Methane Reservoir Engineering. Penn Well Corporation, Oklahoma.

Shen, C.F., Guiot, S.R., 1996. Long term impact of dissolved O_2 on the activity of anaerobic granules. Biotechnol. Bioeng. 49 (6), 611-620. doi:10.1002/(SICI)1097-0290(19960320)49:6<611::AID-BIT2>3.0.CO;2-R.

Shuler, M.L., Kargi, F., 2002. Bioprocess Engineering: Basic Concepts, second ed. Prentice Hall PTR, New Jersey.

Sinha, N., Nepal, S., Kral, T.A., Kumar, P., 2015. Growth and survivability of methanogens at high pressure and high temperature: Implications for subsurface life on Mars, in: 46th Lunar and Planetary Science Conference. Woodlands, Texas, U.S. March 16-20th. https://www.hou.usra.edu/meetings/lpsc2015/ (accessed 12.11.2016).

Sparks, T., 2014. Ceramic proppant design for in situ microbially enhanced methane recovery. Funding Opportunity FOA Number: DE-FOA-0001095. https://www.netl.doe.gov/research/coal/project-information/proj?k=FE0024088 (accessed 15.7.2015).

Strapoc, D., Mastalerz, M., Dawson. K., Macalady, J., Callaghan, A., Wawrik, B., Turich, C., Ashby, M., 2011. Biogeochemistry of microbial coalbed methane. Annu. Rev. Earth Planet. Sci. 39, 617-656. https://doi.org/10.1146/annurev-earth-040610-133343.

Ulrich, G., Bower, S., 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. Int. J. Coal Geol. 76, 25-33. https://doi.org/10.1016/j.coal.2008.03.006.

Valentine, D.L., 2002. Biogeochemistry and microbial ecology of methane oxidation in anoxic environments: A review. Antonie Van Leeuwenhoek. 81, 271-282. doi:10.1023/A:1020587206351.

Waldron, P.J., Petsch, S.T., Martini, A.M., Nusslein, K., 2007. Salinity constraints on subsurface archaeal diversity and methanogenesis in sedimentary rock rich in organic matter. Appl. Environ. Microbiol. 73 (13), 4171-4179. doi:10.1128/AEM.02810-06.

Wawrik, B., Mendivelso, M., Parisi, V.A., Suflita, J.M., Davidova, I.A., Marks, C.R., 2012. Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. FEMS Microbiol. Ecol. 81, 26-42. doi:10.1111/j.1574-6941.2011.01272.x.

Williams, R., Crawford R., 1985. Methanogenic bacteria, including an acid-tolerant strain from peatlands. App. Environ. Microbiol. 50, 1542-1544. http://www.ncbi.nlm.nih.gov/pmc/articles /PMC238796/.

Wolfe, R.S., 2011. Techniques for cultivating methanogens. Methods Enzymol. 494, 1-22.

doi:10.1016/B978-0-12-385112-3.00001-9.

Wray, A., Pashin, J.C., Bustos, O.A., Boyer, C., Lambert, S.W., Johnston, S., Al-Jubori, A., 2009. Coalbed methane: Clean energy for the world. Schlumberger, Oilfield Review Summer. 21, No.2. https://www.slb.com (accessed 12.11.2016).

Xiao, D., Peng, S., Wang, B., Yan, X., 2013. Anthracite bio-degradation by methanogenic consortia in Qinshui Basin. Int. J. Coal Geol. 116-117, 46-52. https://doi.org/ 10.1016/j.coal.2013.06.008.

Zhang, J., Liang, Y., Pandey, R., Harpalani, S., 2015. Characterizing microbial communities dedicated for conversion of coal to methane in situ and ex situ. Int. J. Coal Geol. 146, 145-154. https://doi.org/10.1016/j.coal.2015.05.001.

Zhang, J., Park, S., Liang, Y., Harpalani, S., 2016. Finding cost-effective nutrient solutions and evaluating environmental conditions for biogasifying bituminous coal to methane ex situ. Appl. Energy. 165, 559-568. https://doi.org/10.1016/j.apenergy. 2015.12.067.

Zieminski, K., Frac, M., 2012. Methane fermentation process as anaerobic digestion of biomass. Afr. J. Biotechnol. 11, 4127-4139. doi:10.5897/AJBX11.054.

Zupančič, G.D., Grilc, V., 2012. Anaerobic treatment and biogas production from organic waste, in: Kumar, S., Bharti, A. (Eds.), Management of Organic Waste. InTech., Rijeka, pp. 1-28. doi:10.5772/32756.

2. RESEARCH OBJECTIVES

The research objectives are to:

1. Develop carbon-degrading microbial consortia from various coal sources and environments characterized by natural methanogenesis. A particular methodology for developing these consortia is presented. The most favorable microbial sources, nutrient amendments, coal substrates, and microbial populations that are able to survive initial atmospheric exposure yet retain anaerobic functionality are selected.
2. Examine the potential application of these microbial consortia under low concentration of nutrient amendment and periodic oxygen exposure. Important considerations for their use in field-scale operations.
3. Evaluate environmental constraints, such as temperature, pH, and salinity (NaCl) on coal biogasification. The aim is to look for the best system performance and the largest methane production with at least one of the microbial consortia.

3. ORIGINAL CONTRIBUTION OF THE RESEARCH

As original contributions, the research will:

- Develop a unique set of microbial consortia that can tolerate periodic oxygen exposure and still exhibit methanogenic activity during coal bioconversion through the implementation of a screening procedure.
- Explore the potential application of the microbial consortia with limited nutrient amendments and periodic oxygen exposure in order to consider their usability for coal biogasification.
- Examine the influence of environmental constrains, such as pH, salinity (NaCl), and temperature on the process of coal bioconversion through the use of fitted empirical models in relation to an experimental design.
- Determine a domain of operation in which methane production from coal can be maximized for a particular case.

4. DEVELOPING METHANOGENIC MICROBIAL CONSORTIA FROM DIVERSE COAL SOURCES AND ENVIRONMENTS

4.1. Abstract

Biogenic gas production is a promising alternative or supplement to conventional methane extraction from coalbeds. Adsorbed and free gas, generated over geologic time, can be supplemented with biogenic gas during short-term engineering operations. There are two generic protocols for doing this. The first is to contact the coal with nutrients to support native bacterial development. The second approach is to inject appropriately cultured ex situ consortia into subsurface coal accumulations. Research has mainly focused on the former: in situ stimulation of native microbial communities with added nutrients. Relatively few studies have been conducted on the strategies for enriching ex situ microbial populations under initial atmospheric exposure for subsequent injection into coal seams to stimulate biodegradation and methanogenesis. To evaluate the feasibility of ex situ cultivation, natural microbial populations were collected from various hydrocarbon-rich environments and locations characterized by biogenic methane production. Different rank coals (i.e., lignite, subbituminous, bituminous), complex hydrocarbon sources (i.e., oil shale, waxy crude), hydrocarbon seeps, and natural biogenic environments were incorporated in the sampling. Three levels of screening (down-selection to high grade the most productive consortia) allowed selection of microbial populations, favorable nutrient amendments, sources of the microbial community, and quantification of methane produced

from various coal types. Incubation periods of up to twenty-four weeks were evaluated at 23°C. Headspace concentrations of CH_4 and CO_2 were analyzed by gas chromatography. After a two-week incubation period of the most promising microbes, generated headspace gas concentrations reached 873,400 ppm (154 sft^3/ton or 4.8 scm^3/g) for methane and 176,370 ppm (31 sft^3/ton or 0.9 scm^3/g) for carbon dioxide. Rudimentary statistical assessments – variance analysis (ANOVA) of a single factor - were used to identify trends and levels of significance or impact of the consortia enrichment. It was demonstrated that microbial communities from coal and lake sediments can be enriched and adapted to effectively generate methane after initial atmospheric exposure. The development and enrichment of these methanogenic consortia is described.

4.2. Introduction

Coalbed methane (CBM) accounts for an important fraction of natural gas reserves in the world; up to 20% of this natural gas has been suggested as having a microbial (biogenic) origin (Ritter et al., 2015). Recognizing this, there is an opportunity for producing additional methane by stimulation of existing microorganisms or by introducing other microorganisms. Various high and low rank methane-producing coal reservoirs are known worldwide. Examples include: The Powder River, San Juan, Illinois, Gulf Coast, Black Warrior, Utah, and Appalachian Basins in the United States. The Hailar-Inner Mongolia, Qinshui Basin, and Ordos Basins are prominent in China. The Bowen, Surat and Sydney Basins are productive in Australia, and the Barmer-Sanchor Basin is a prominent coal region in India (Faiz and Hendry, 2006; Green et al., 2008; Li et al., 2016; Park and Liang, 2016; Rathi, et al., 2015; Ritter et al., 2015; Strapoc et al., 2011; Xiao et

al., 2013).

Methanogenic stimulation and gas production could be of particular interest after primary production of geologically adsorbed gas has substantially declined. Alternatively, waste heaps near coal mines could quite simply be processed at the surface. As a potential resource and with an already established recovery, transportation, and processing infrastructure, biogenic methane production might be considered as an important supplement to conventional methane extraction from coalbeds.

Different techniques for microbially enhanced coalbed methane production are known (Park and Liang, 2016). Among them, microbial augmentation (i.e., adding new or additional microorganisms to the coal in order to enhance or initiate microbial CBM production) emerges as a promising alternative. Even so, there remains uncertainty about the implementation of microbial consortia, their effectiveness, and their sustainability. There are also significant knowledge gaps related to performance and effective operations (Park and Liang, 2016; Ritter et al., 2015).

In recent years, various investigators have focused on the enhancement of biogenic methane production from different rank coals. Fallgren et al. (2013), for instance, evaluated four coal types. Among them, a lignite (Beulah-zap), a subbituminous coal (Wyodak-Anderson), a high-volatile (HV) bituminous coal (Pittsburgh No. 8), and a low-volatile (LV) bituminous (Pocahontas No. 3) coal were considered. Green et al. (2008) used a subbituminous B Wyodak coal as substrate. Gupta and Gupta (2014) evaluated a Hard coal. Harris et al. (2008) investigated lignite and subbituminous coals. Jones et al. (2010) considered a subbituminous coal. Opara et al. (2012) evaluated bituminous coal, waste coal and a North Dakota lignite sample. Menger et al. (2000) only reported the use of lignite as

coal type. Pfeiffer et al. (2011) considered the stimulation of biogenic gas from lignite, subbituminous and bituminous coals. Papendick et al. (2011) evaluated subbituminous Walloon coal as the primary carbon source. Rathi et al. (2015) investigated bituminous coal as carbon source. Ulrich and Bower (2008) used subbituminous coal as substrate. Zhang et al. (2015) and Zhang et al. (2016) investigated bituminous coal. Wang et al. (2017) studied coal biogasification from lignite coal samples. Most published studies involve attempts to enhance microbial activity and methane production through in situ stimulation of native microbial populations. Conversely, microbial augmentation has received less attention (Park and Liang, 2016).

Although uncertainties related to microbial augmentation or bioaugmentation require consideration, some research groups and corporations currently have proceeded aggressively (Jones et al., 2010; Opara et al., 2012; Pfeiffer et al., 2011; Scott and Guyer, 2004; Srivastava, 1997). ARCTECH, Inc., for instance, has adapted microorganisms derived from wood-eating and humus-eating termites for biogenesis in coal with selected nutrient compounds (Ritter et al., 2015).

As an important element for microbial functioning, nutrient amendments must be considered. Meet the microorganisms' needs is required to promote their growth and metabolic activities which include degrading coal to methane (Park and Liang, 2016). Different nutrient solutions have been tested (Park and Liang, 2016; Zhang et al., 2015). Generally, these solutions are composed by major minerals (e.g., K, Na, Ca, Mg, NH_4^+, P and Cl) supplied at concentrations of g/L. Other ingredients that include organic nitrogen and vitamins sources such as yeast extract, peptone or tryptone are also used at g/L level. Vitamin solutions which may contain more than 10 vitamins and trace mineral solutions

are added at less than mg/L concentrations (Bao et al., 2016; Park and Liang, 2016). Research has shown that different media can have a dramatically different effect on methane production (Zhang et al., 2015). Thus, for a given coal sample and a microbial community, testing different solutions may be beneficial if the goal is to increase methane productivity (Park and Liang, 2016).

Opara et al. (2012) and Opara (2012) conducted one of the first studies related to the development of methanogenic microbial consortia that, after being initially exposed to the atmosphere, still proceed with methanogenesis. An initial enrichment step and subsequent evaluation of gas production from selected coal and waste coal sources were considered. Zhang et al. (2015) also worked on the development of microbial consortia and indicated the possibility of culturing under conditions that are not strictly anaerobic.

Following a strategy of mainly evaluating methane and carbon dioxide production from coal, the present work adopted a related but more complex methodology to develop methanogenic microbial consortia. This methodology included the recollection and enrichment of microbial populations from various hydrocarbon-rich environments and locations characterized by natural methanogenesis. The selected sources of microbial communities included different rank coals, complex hydrocarbon materials (i.e., oil shale, waxy crude), hydrocarbon seeps, and natural biogenic environments. Three screening phases allowed to select the most favorable microbial sources, nutrient amendments, coal substrates, and microbial populations. With each phase of screening, favorable microbial samples were high graded. In the experimental program described herein, promising microbial communities were obtained after three screening phases which involved a continuous enrichment with fresh nutrient amendments and a final adaptation stage

conditioning these consortia to specific coal types.

The aim of this study has been to document the development and enrichment of microbial consortia that can be relevant for methane production in in situ and ex situ scenarios. In addition, it is expected that this work leads to in-depth research on development of methanogenic consortia under incomplete anaerobic conditions. Our results suggest that reducing agents (e.g., Na_2S, cysteine-HCl), and inert gases (e.g., nitrogen, argon) should not be used to initially flush bioreactors to remove oxygen, and allowing the cultured microbes to evolve under initial atmospheric exposure; an important consideration for their use in field-scale operations, as maintaining strict anaerobic conditions would be difficult and expensive. The delivery of the microorganisms into subsurface reservoirs will likely involve some atmospheric exposure that can limit the methane production from strict anaerobes.

4.3. Materials and Methods

4.3.1 Coal and other Hydrocarbon Samples

Different rank coal samples were provided by the Industrial Combustion and Gasification Research Facility at the University of Utah. These samples, characterized in Table 2, are designated as East Texas, Miller Black Thunder, NARM Cook, North River, Illinois #6, Red Hills, Utah Skyline, Arkansas, and two samples from the Deer Creek Mine in Utah (a coal sample from a waste pile at the coal mine and a soil sample near the waste pile). Proximate and ultimate analyses of these coals are shown in Table 2 and Table 3, respectively. Based on these analyses and published information, the coal rank was established (Hecht et al., 2013; Opara, 2012; Opara et al., 2012; Prior et al., 1985; Sarv et

al., 2009; Speight, 1983, 2005; Vaysman and Yixin, 2012; Wise, 1990). Coal rank can depend on volatile matter, fixed carbon, inherent moisture, and oxygen. No single parameter determines its classification. Generally, the coal rank increases with the amount of fixed carbon, but decreases with the amount of volatile matter and moisture. For example, subbituminous coals can have higher moisture, and lower sulfur, fixed carbon content, and lower heating values than bituminous coals (Prior et al., 1985; Speight, 1983, 2005; Wise, 1990).

Other hydrocarbon samples were also studied. These included an oil shale sample (moderately kerogen-rich carbonate). This sample was obtained from the White River Mine in the Uinta Basin in eastern Utah. Additionally, a highly paraffinic oil sample, colloquially known as "waxy crude" because of their low pour points was used.

The coal samples and the waste coal sample from the Deer Creek Mine were received as pulverized material (< 0.42 mm particle size), thus providing a large surface area. The associated soil sample of Deer Creek Mine was left unaltered. The oil shale sample was crushed, and the paraffinic crude was untreated. This paraffinic crude has a pour point of approximately 41°C. This means that it is a viscous semi-solid at the incubation temperature of nominally 23°C. It is acknowledged that particle size of coal has an important effect on the extent of biological methane production (Green et al., 2008; Gupta and Gupta, 2014), and the results presented here represent a desirable scenario.

Smaller coal particle size is related to bigger coal surface area. Large surface area would facilitate microbial colonization; therefore, more methane generation would be expected than using large particle sizes (Bao et al., 2016). The extrapolation of gas production rates to in situ operations can be carried out (Green et al., 2008). However, the

effects of other important factors (e.g., pressure, temperature, salinity) should be also taken into account to replicate truly in situ conditions (Bao et al., 2016; Park and Liang, 2016). All samples were exposed to air during storage, handling, and preparation. This may have influenced their bioavailability and biodegradability. In fact, this exposure is an important consideration taking into account the desirability of culturing effective methanogens in an aerobic field scale production scenario.

4.3.2. Environmental Samples

Recognizing the occurrence of natural biogenesis, sediments were collected from locations where near-surface methane and/or hydrocarbon would likely be present. One of these locations was Rozel Point on the shore of the Great Salt Lake, Utah. Samples were collected from various surface oil seeps at Rozel Point. Other samples were obtained from the marshes of Utah Lake and the Jordan River riverbed near the outlet of Utah Lake. The Utah Lake samples were collected in a marsh near Saratoga Springs, Utah.

4.3.3. Media Types for Culturing of Microbes

Aseptic techniques were used to enrich microbes from the collected samples. These included sterilization of tools and solutions in an autoclave (121°C, 25 min), disinfection of sample station (i.e., microbiology hood) with 10% Chlorox solution before and after work, turning UV light under the hood for 30 min, use of gloves in collecting samples. Five nutrient media were used. These incorporated ingredients such as acetate (i.e., direct methane precursor), phosphate (i.e., source of phosphorous), urea (i.e., source of nitrogen), and a balanced nutrient solution of tryptic soy broth. Preliminary studies have shown

favorable results for the ex situ cultivation of aero-tolerant methanogenic microbial consortia when using these nutrient amendments (Opara, 2012). Thus, they were considered for this work. The specific nutrient media compositions are:

- acetate - ACE (3.5 g/L sodium acetate);
- tryptic soy broth - TSB (15 g/L);
- acetate-yeast-phosphate medium - AYP (2.5 g/L sodium acetate, 0.75 g/L yeast extract, and 0.5 g/L potassium phosphate monobasic);
- yeast-urea-phosphate medium - YUP (1.25 g/L yeast extract, 0.15 g/L urea, and 0.5 g/L potassium phosphate monobasic) and,
- lactate medium - LAC (1 g/L yeast extract, 6.67 mL/L sodium lactate, 1.23 g/L sodium acetate, 0.5 g/L ammonium chloride, 1 g/L potassium phosphate, 0.2 g/L magnesium sulfate, 0.1 g/L calcium chloride, and 0.5 g/L sodium sulfate).

These nutrient media provided easily degradable carbon sources and appropriate nutrient compounds (e.g., nitrogen, phosphorous) to stimulate microbial growth and methane production (Opara, 2012). No pH buffer was used, and all of the nutrient media were close to a neutral pH. Additionally, it is noted that acetate can mainly favor the enrichment of acetoclastic methanogens, and/or microorganisms able to use acetate as part of their metabolic functions (e.g., syntrophic acetate oxidizers) and likely provide some H_2/CO_2 to hydrogenotrophic methanogens (Meslé et al., 2013).

4.3.4. Experimental Setup

Different levels of screening were sequentially conducted to engineer a diverse set of microbial consortia that can be used to degrade coal and proceed with methanogenesis.

These screening procedures allowed for down-selection of favorable microbial sources, nutrient amendments and microbial populations on the basis of methane and carbon dioxide production.

During experimentation, the headspace of the bioreactors was not initially sparged with nitrogen or argon gas. This allowed the cultured microbes to evolve under initial atmospheric exposure. All enriched consortia were exposed to the atmosphere during the collection and cultivation steps. These initial aerobic conditions were intentionally chosen to develop microbial consortia throughout the experimental program. This demonstrated the ability for microbial consortia to survive and produce methane at low to moderate oxygen concentrations: a key consideration for their use in field-scale operations. The maintenance of strict anaerobic conditions can be difficult and expensive. The delivery of the microorganisms into subsurface reservoirs will likely involve some atmospheric exposure that could limit the methane production from strict anaerobes.

All samples were kept at 23°C to emulate the temperature of the natural habitat where microbial populations were collected. Sterile, 50-mL centrifuge tubes were used as the bioreactors in all phases of the screening program. These were set aside without agitation over the prescribed reaction periods. Two-hundred microliters (200 µL) of produced gas were directly extracted under sterile conditions using a gas-tight syringe (Hamilton Co., GASTIGHT® #1750) through a small hole in the caps that were completely covered with silicone gel. Silicone was periodically reapplied to the end caps to prevent leakage (Fallgren et al., 2013). In addition to this precautionary measure, the bioreactors can be visually checked by spreading a solution of gas leak detector and checked for bubble generation (ASTM E515-05). Methane production was monitored in the headspace of the

bioreactors using gas chromatography.

Carbon dioxide was also measured because the carbon dioxide itself can be a valuable byproduct in situ, and preferential CO_2 adsorption may actually enhance methane recovery from treated coal. There certainly is experience for preferential production of geologically-generated, adsorbed methane during carbon dioxide injection into coalbeds (Smith, 2010; White et al., 2005). Since effective mesophilic methanogens can have longer regeneration times and slower growth than others microorganisms involved in the biotransformation of complex organic matter, long periods of incubation were evaluated (Del Real, 2007; Ferry, 1993; Zupančič and Grilc, 2012).

Unless specified, control samples were created by adding sterile saline solution - 8.5 g/L NaCl - to the respective coal samples and sediments. Gases measured from these controls were hypothesized to be residual gas desorption and/or generation by indigenous microbes (Opara et al., 2012; Opara, 2012). It is conceived that microorganisms need salts to function. Salts provide essential elements (e.g., sodium, potassium) for the formation of new cells (Liu and Boone, 1991; Schnürer and Jarvis, 2010). Freshwater methanogens, for instance, need at least 2.3×10^{-2} mg/cm^3 of sodium for their growth and metabolic functions (Ferry, 1993; Megonigal et al., 2013; Patel and Roth, 1977). These control samples were initially included to compare and evaluate the effectiveness of using select nutrient amendments to promote microbial growth and methane production during experimentation. Moreover, for practical and comparative purposes the overall conversion of nutrient amendments to methane and carbon dioxide was estimated using the following equation (Buswell and Neave, 1930; Kayhanian et al., 2007; Opara, 2012; Opara et al., 2012; Wagner et al., 2012; Wang et al., 2017):

$$C_cH_hO_oN_nS_s + yH_2O \rightarrow \mathrm{x}CH_4 + (c-x)CO_2 + nNH_3 + sH_2S \quad (1)$$

where x = 0.125(4c+h-2o-3n-2s), and y = 0.25(4c-h+2o+3n+2s)

4.3.4.1. Screening Protocol Phase I

The objective of this first screening phase was to select samples that could produce significant amounts of methane and carbon dioxide. Also, samples that showed unusual qualitative features, such as darkened solution from apparent breakdown of coal after enrichment, were selected for additional evaluation.

Five gram aliquots of coal or sediment samples were placed separately into bioreactors. Thirty milliliters of nutrient medium were added. An initial set of five nutrient amendments was considered. Subsequently, some nutrient media would be selected on the basis of the gas production for the next phase of screening. Duplicate samples were prepared for each coal and sediment aliquot with each nutrient medium. As an example, 5 g of Miller Black Thunder – MBT coal were placed in each of twelve bioreactors; ten of these bioreactors contained coal plus a nutrient medium, and the remaining two bioreactors contained coal plus saline solution - 8.5 g/L NaCl- as control samples. The amounts of sample and nutrient medium were chosen so that about one third of the total volume corresponded to headspace. Samples were incubated for up to eight weeks. Measurements of headspace gas were taken after two and eight weeks.

4.3.4.2. Screening Protocol Phase II

The objectives of this phase of screening were to keep selected microbial consortia actively stimulated by introducing nutrients for a second time and to refine and select microbial samples that would produce significant amounts of methane and carbon dioxide. Three bioreactors were used and these contained acetate – ACE, tryptic soy broth – TSB, and lactate – LAC media, respectively. These nutrient media were chosen considering the positive stimulation observed in the preliminary phase of screening, and were used to continue the enrichment of microbes and gas production in this phase. Each bioreactor contained 10 mL of the selected sample, in two 5 mL aliquots, and 20 mL of fresh nutrient solution. These samples were set aside for fourteen days. After that time, the liquid media were turbid and bubble generation was observed, suggesting possible microbial growth.

4.3.4.3. Screening Protocol Phase III

The objective of this final phase was to continue the enrichment of the microbial consortia with fresh nutrient media. To finally select promising methanogenic populations, the cultivated consortia were allowed to adapt to selected coal samples of different rank. Microbial samples were mixed with four coal types and three nutrient media (acetate – ACE, tryptic soy broth – TSB, and lactate – LAC). These nutrient media corresponded to the same type used in the previous phase of screening, and were chosen to continue promoting the growth and metabolic activities of the microbial populations. The four coal types were subbituminous Miller Black Thunder – MBT, bituminous Deer Creek Mine Waste coal – DCWC, high volatile bituminous Praxair Illinois #6 – PI, and Arkansas Lignite – LIG. These coal samples were chosen in order to obtain microbial consortia able

to use different rank coals and to select the most favorable coal substrates. Five grams of the selected coal types and 20 mL of nutrient media were combined, resulting in twelve bioreactors plus controls for each coal type with added nutrients.

Twelve bioreactors were used for samples from the previous screening phase. They are denoted according to the nutrient medium added. For instance, 20 mL of acetate – ACE medium and 5 mL of sample RH TSB ACE were added into each of four bioreactors containing the four selected coal types. Alternatively, four bioreactors containing the coal types were mixed with 20 mL of tryptic soy broth – TSB and inoculated with 5 mL of RH TSB TSB sample. The remaining four bioreactors that were mixed with 20 mL of fresh lactate – LAC medium included 5 mL from the RH TSB LAC sample. Incubation periods were monitored for up to twenty-four weeks. Gas measurements were performed after two weeks and twenty-four weeks of incubation.

As mentioned above, three screening phases were carried out to select the most favorable microbial sources, nutrient amendments, microbial consortia, and to identify prolific (in terms of methanogenesis) coal substrates. After each phase of screening, consortia were down-selected for the next phase. A flow diagram that summarizes the described screening protocols is shown in Figure 2.

4.3.5. Aqueous Extraction of Coal Samples

Deionized water was used as a solvent to extract some water-soluble compounds (i.e., polar compounds) from the structure of coal samples, such as low molecular weight organic acids, amino acids, and some alcohols (Furmann, 2011). Twelve milliliters of DI water and 2.4 g of coal samples were added into glass centrifuge tubes (Kimble, Cat No.

73785-15). These tubes were intermittently mixed during six days at room temperature (23°C). Three extraction cycles were used, each cycle corresponded to two days long. Aqueous extracts were kept under refrigeration at 4°C prior analysis. These experiments were considered to identify possible inhibitory and polar compounds present in the coal sources tested.

4.3.6. Stability of Microbial Consortia in Anaerobic Conditions

In order to assess whether or not the microbial consortia were truly both aero-tolerant while being anaerobically methanogenic, an additional experiment was conducted. The combination of both aero-tolerance and anaerobic methanogenesis is ideal from a biogasification standpoint. It is unlikely that a process restricting complete air contact would be viable (Bum-Han et al., 2017; Opara 2012; Opara et al., 2012; Zhang et al., 2015).

To create an initial anaerobic condition, the promising microbial sample RH TSB TSB MBT was placed in an argon environment (glovebox). This sample was then sealed with silicone gel in a 50-mL bioreactor of the same type previously used during the screening protocols. Another bioreactor was simultaneously prepared under initial atmospheric exposure. Five grams of the selected coal type (i.e., subbituminous Miller Black Thunder – MBT, < 0.42 mm particle size) and 20 mL of nutrient media (i.e., tryptic soy broth – TSB) were used. Subsequently, 5 mL of inoculum were washed and added into each bioreactor. Headspace methane concentration was monitored up to three weeks of incubation. The bioreactors were set aside without agitation at 23°C.

4.3.7. Gas Chromatography

The headspace methane and carbon dioxide concentrations in each bioreactor were determined with a Hewlett Packard HP6890 GC system (Palo Alto, CA) with a GS-GasPro PLOT column containing a bonded silica-based stationary phase. A flame ionization detector (FID) and thermal conductivity detector (TCD) were connected in series to analyze organic compounds and inorganic gases. Helium was used as the gas carrier. The temperature program used for this study began with 35°C for 4 min to allow for carbon dioxide and methane elution and was then increased by 25°C min^{-1} to 260°C. Scotty Analyzed Gases were used as standards to build calibration curves for methane and carbon dioxide. GC ChemStation (Agilent Technologies) computer software was used.

4.3.8. Gas Chromatography and Mass Spectrometry

High-performance liquid chromatography (HPLC) was initially considered to identify compounds in aqueous extracts, which were initially filtered by gravity using a glass microfiber (0.7 μm Whatman GF/F prebaked at 500°C). However, dissolved compounds were not identified. Thus, a Shimadzu GCMS-QP2010 Ultra equipped with a 30 m×0.25 mm i.d., 0.25 μm dF Restek Stabilwax column was subsequently used. The GC-MS program is described as follows: temperature of 50°C raised to 250°C with rate of 25°C/min, holding time of 10 min, pressure 57.4kPa, total flow 12.1 mL/min, linear velocity 37.2 cm/sec, purge flow 0.5 ml/min, split ratio 10.0. GC-MS data were acquired, processed and evaluated using GCMS solution software. Compound identification was carried out using residence time and mass spectrum (scan range of m/z from 35 - 300) according to the 2008 version of the NIST Standard Reference Database.

4.3.9. Statistical Analysis

Microbial consortia development trends were examined using the statistical software package, STATGRAPHICS Centurion VII®. The variance analysis ANOVA of a single factor was used to evaluate the 288 samples that came from Phase III screening. These samples were chosen since all of the factors related to the experimental matrix were included (the term "factors" refers to experimental variables that can be changed independently of each other, and the term "levels" refers to different values or categories within the factor for which the experiments were carried out). Factors and levels for this study are summarized in Table 4.

The statistical Kruskal-Wallis test was used to evaluate populations that did not have a normal distribution. This tests the hypothesis that medians of all levels are equal within their corresponding factor. Results or measured values of all levels were combined and ordered from the lowest to the highest, assigning a rank. Subsequently, an average rank was computed for each level, and a p-value was used to evaluate the hypothesis. High and low significances for the methane and carbon dioxide production at two and twenty-four weeks were identified by comparing the average rank, median or mean values (Kruskal and Wallis, 1952; Lizasoain and Joaristi, 2003).

4.4. Results and Discussion

Headspace concentrations of methane and carbon dioxide were monitored at defined intervals in each phase of the screening program. However, only limited conclusions can be drawn about the bioconversion kinetics from these results. It is not known when or whether maximum gas production was achieved. Considering the long

regeneration times, slow growth of mesophilic methanogens enriched in the cultures and the large number of microbial samples generated during experimentation, long intervals for sampling headspace gas were carried out.

Gas concentrations are reported in parts per million (ppm), and approximate values of sft^3/ton of coal or sediment were determined (Opara, 2012; Opara et al., 2012). Units of sft^3/ton or scm^3/g are meaningful units for assessing commercial viability of coalbed methane gas production (Park and Liang, 2016). Over 800 samples were created during the full screening program. Many microbial samples did not generate significant methane and carbon dioxide concentrations during experimentation. Results for the microbial samples that produced the highest methane and carbon dioxide concentrations are included in this work. The samples reported have been labeled according to the sequence of the screening program and the abbreviations previously shown in Table 2.

4.4.1. Phase I Screening

4.4.1.1. Hydrocarbon Samples

At two weeks of incubation, methane concentrations less than 4,800 ppm (0.8 sft^3/ton or $2.6x10^{-2}$ scm^3/g) were measured for most of the evaluated coal samples. Control samples showed much lower concentrations - up to 25 ppm ($4.1x10^{-3}$ sft^3/ton or $1.4x10^{-4}$ scm^3/g). On the other hand, during this initial 14-day period, carbon dioxide production was significant. For example, up to 400,000 ppm (71 sft^3/ton or 2.2 scm^3/g) were measured with lactate – LAC medium and the subbituminous Miller Black Thunder – MBT coal (i.e., MBT – LAC2 in Figure 3). Modest carbon dioxide concentrations up to 76,000 ppm (13.5 sft^3/ton or 0.4 scm^3/g) were also measured from the control samples. The occurrence of

these carbon dioxide concentrations from the different coal rank samples and nutrient media likely indicated rapid stimulation of microbial populations, despite the small volumes of methane production. However, it is very likely that some of the generated gas came from nutrient amendments during this period of incubation.

After eight weeks of incubation, significant methane was generated in the bioreactors that included coal samples. For instance, methane concentrations up to 640,000 ppm (113 sft^3/ton or 3.5 scm^3/g) were measured for Deer Creek Mine Waste coal with lactate media (i.e., DCWC LAC2 in Figure 3), while concentrations less than 256 ppm ($4.5x10^{-2}$ sft^3/ton or $1.4x10^{-3}$ scm^3/g) were determined from the control samples (Table 5). This suggests that commercially viable methane production is possible.

The bioconversion of complex organic substrates into methane is thought to incorporate multiple processes. These include solubilization, hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Interactions among various microbial communities take place, and the processes are characterized by different growth rates and metabolic functions (Lema et al., 1991; Schink, 2006; Senthamaraikkannan et al., 2016; Shuler and Kargi, 2002; Strapoc et al., 2011). Thus, eight weeks of incubation were used to positively stimulate microbial growth of species present in coal sources and to proceed with methanogenesis.

The low rank coal samples, such as Red Hills – RH and East Texas – ETL mixed with TSB and lactate – LAC (i.e., RH TSB1, ETL TSB1, and ETL LAC2 in Figure 3) were among the highest methane producers at eight weeks. These samples probably provided significant microbial populations that were stimulated with the added nutrients. Alternatively, these coal sources were likely more susceptible to biodegradation due to

their lower rank, permeability, and the fact that there were highly branched compounds accessible to active microorganisms (Fallgren et al., 2013; Strapoc et al., 2011; Wise, 1990). Carbon dioxide concentrations, on the other hand, were lower: CO_2 concentrations were less than 230,000 ppm (40.5 sft^3/ton or 1.3 scm^3/g) for the coal samples after eight weeks.

When other potential methanogenic sources were evaluated – such as the oil shale sample – lower methane concentrations were observed. The highest methane concentration was 160 ppm ($2.8x10^{-2}$ sft^3/ton or $8.8x10^{-4}$ scm^3/g) while 38 ppm ($6.7x10^{-3}$ sft^3/ton or $2x10^{-4}$ scm^3/g) was measured from the control sample. This oil shale produced nearly 220,000 ppm (39 sft^3/ton or 1.2 scm^3/g) carbon dioxide, which was significant higher than 20,000 ppm (3.5 sft^3/ton or $11x10^{-2}$ scm^3/g) produced by the control sample (salt solution plus oil shale sample). No appreciable methane production (i.e., concentrations less than 13 ppm, $2.3x10^{-3}$ sft^3/ton or $7.1x10^{-5}$ scm^3/g) was generally detected for the waxy crude samples and controls. The highest carbon dioxide concentration was nearly 94,000 ppm (16.5 sft^3/ton or $9.7x10^{-1}$ scm^3/g) compared to 1400 ppm ($2.4x10^{-1}$ sft^3/ton or $7.7x10^{-3}$ scm^3/g) from control sample. Apparently, few appropriate methanogenic populations were present in those samples. In addition, bacteriostats and bactericides could be naturally present, or may have formed as a result of biochemical reactions inhibiting or destroying the molecular cell structure of remaining microbes (Wise, 1990). Further studies need to be conducted in order to evaluate the use of other hydrocarbon samples, such as those considered here (i.e., oil shale and waxy crude), as alternative sources of microbial populations suitable for coal biotransformation.

4.4.1.2. Environmental Samples

Significant methane production was measured for the environmental samples acquired from the Jordan River – JR and Utah Lake – UL sediments, even after only two weeks of incubation (e.g., JR1 ACE2, UL6 ACE2 in Figure 3). These samples showed methane concentrations much larger than those for samples from Great Salt Lake (GSL) sediments (i.e., 650 ppm, 0.11 sft^3/ton or $3.5x10^{-3}$ scm^3/g). This may be due to more microbial population or high activity of degrading organic matter present in sediments. Microbial populations can be positively stimulated after nutrient addition (Opara et al., 2012). This likely overcame the lack of essential nutrients initially present to support methanogenesis. These samples produced methane concentrations up to 208,800 ppm (37.2 sft^3/ton or 1.1 scm^3/g) (e.g., UL6 ACE2 in Figure 3). These methane concentrations were significantly higher than any control sample (71.3 ppm, $1.26x10^{-2}$ sft^3/ton or $4x10^{-4}$ scm^3/g) (Table 5) and the estimated maximum methane production from added nutrients (Table 6). Carbon dioxide concentrations up to 400,000 ppm (71 sft^3/ton or 2.2 scm^3/g) were measured for the evaluated samples (e.g., UL4 TSB2 in Figure 3), and 6,940 ppm (1.2 sft^3/ton or $3.7x10^{-2}$ scm^3/g) was measured for the control samples (Table 5).

Methane and carbon dioxide concentrations increased when nutrient amendments were added to samples from hydrocarbon seeps adjacent to Great Salt Lake. Lower concentrations (70 ppm, $1.2x10^{-2}$ sft^3/ton or $3.8x10^{-4}$ scm^3/g) were generated from their control samples. It may be inferred that the Great Salt Lake sediments had low concentrations of easily degradable organic matter and/or relevant nutrients required to support microbial activity. In addition, it is suspected that the high salt concentration, characteristic of Great Salt Lake's environment – with NaCl concentrations that can exceed

250 g/L (Naftz et al., 2011; Sturm, 1980), was detrimental to methane production. The inhibitory role of salinity was confirmed in subsequent unpublished studies by the authors and has been reported by other researchers (Head et al., 2014; Liu et al., 1991; Patel and Roth, 1977; Rathi et al., 2015).

Figure 3 summarizes the microbial samples chosen from the Phase I screening. For illustrative purposes only the best methane and carbon dioxide producers are shown. These samples were organized from the highest to the lowest methane concentration in order to show the most promising samples. It is noted that duplicate microbial samples are included in the figure. For example, UL4 TSB1 and UL4 TSB2 correspond to the environmental sample Utah Lake – UL combined with tryptic soy broth – TSB. Microbial samples include the highest methane producers after an eight-week incubation period, the highest methane producers after two weeks in an acetate medium, as well as the highest carbon dioxide producers after two and after eight weeks.

Also shown are samples that were chosen subjectively on the basis of their appearance after eight weeks (e.g., samples with visible bubble generation). These latter samples exhibited features that were presumed to be a consequence of coal breakdown and methanogenic activity. Results associated to controls of Figure 3 are presented in Table 5. Additionally, theoretical conversion of added nutrients to methane and carbon dioxide was estimated (Buswell and Neave, 1930; Kayhanian et al., 2007; Opara, 2012; Opara et al., 2012; Wagner et al., 2012; Wang et al., 2017). These results suggest that most of generated gases came from the digestion of coal and/or sediments, and just a small fraction could be attributed to the nutrient amendments used. These values are shown in Table 6. Over seventy-five samples were selected for further refinement and subsequent evaluation.

4.4.2. Phase II Screening

During this phase of screening, nutrient amendments were again added (i.e., acetate – ACE, lactate – LAC, and tryptic soy broth – TSB). This ensured that the microbial populations remained actively stimulated. This phase can also be considered as a preliminary step for the ensuing final stage of screening. Figure 4 shows the best methane producers and their associated carbon dioxide concentrations after two weeks of incubation. The results were very promising. For example, 633,000 ppm (111 sft^3/ton or 3.4 scm^3/g) methane were measured for sample MBT LAC ACE (Miller Black Thunder coal amended with lactate – LAC in Phase I and acetate – ACE in Phase II).

In Phase II screening, the highest methane producers correlated with specific coal sources, mainly East Texas lignite – ETL and the subbituminous Miller Black Thunder – MBT (Figure 4). This might indicate that active microorganisms were continuously stimulated with new nutrients that either were depleted or were not present in Phase I screening after eight-week incubation period. Enrichment with fresh nutrients could have met the nutritional requirements of the microbial populations, affording growth and promoting methane production from remaining coal and/or sediments. Low gas concentrations from added nutrients were estimated (Table 7). This suggests that nutrient solutions effectively stimulated microbial consortia.

On the other hand, some samples such as the Utah Lake – UL and Jordan River – JR sediments manifested a different behavior (Figure 4). For these sediments, the stimulated microbial populations continued producing significant amounts of carbon dioxide, as had been previously observed during the Phase I screening (Figure 3).

As seen in Figure 4, samples that included microbial communities from different

rank coals and environmental sources were satisfactorily stimulated. However, not all of the nutrient amendments were equally effective for methane production. Nitrogen and phosphorous present in lactate – LAC and tryptic soy broth – TSB media are considered essential for simulating methane generation from coal (Jones et al., 2010; Ritter et al., 2015). Since different compounds were present in these nutrient amendments, it may be advantageous to identify the exact stimulants that impacted the overall performance of the microbial consortia. Once their role is identified, simplified and less expensive nutrient amendments can be used for enhancing methane yields from coal (Zhang et al., 2016).

4.4.3. Phase III Screening

Fresh nutrient media and selected coal types were used to sequentially enrich and adapt microbial populations that had been down-selected from Phase II screening after two-week incubation period. This included four different rank coals (subbituminous Miller Black Thunder – MBT, bituminous Deer Creek Mine Waste coal – DCWC, high volatile bituminous Praxair Illinois #6 – PI, and Arkansas Lignite – LIG) and the same nutrient media used in the previous phase of screening. Methane and carbon dioxide were monitored after two and twenty-four weeks of incubation.

Figure 5 summarizes the premium methane producers at two weeks of incubation. In addition, results of theoretical gas production from the fresh nutrient amendments and controls related to the evaluated samples are included in Table 7 and Table 8, respectively. It is noted that the same amount and type of nutrient amendments were used in Phase II and Phase III of screening. Only a small fraction of methane and carbon dioxide can be attributed to direct result of nutrient conversion from the microbial samples amended with

the selected nutrients (Table 7).

The maximum methane concentration of 873,000 ppm (154 sft^3/ton or 4.8 scm^3/g) was measured for one of the most promising samples JR2 TSB TSB DCWC. The carbon dioxide concentration for this sample was 176,370 ppm (31 sft^3/ton or 0.9 scm^3/g). Among the samples that included microbial populations enriched from coal sources, the lignitic Red Hills – RH and East Texas – ETL coals showed significant methane production. Large methane concentrations were also measured with microbial populations retrieved from the lake sediments (e.g., Jordan River – JR, Utah Lake – UL in Figure 5). For each sample, an optimal microbial population, nutrient amendment and/or coal combination were obtained.

Significant methane production was evident for the higher rank coals used for adaptation in this phase of screening (i.e., bituminous Deer Creek Mine Waste coal – DCWC, subbituminous Miller Black Thunder coal – MBT in Figure 5). For those coal samples, relatively lower methane production was observed in comparison to the lower rank coals in previous phases of screening. This observation of low methane production from the higher rank coals during the initial stages of screening, followed by higher methane concentrations in this phase of screening can be related to the positive stimulation of methanogenic populations after subsequent enrichment, and can indicate less inhibition of ex situ cultured microbial populations with higher rank coals rather than their lower-rank counterparts.

Bituminous coal is generally characteristic of greater depths in a reservoir (Rathi et al., 2015), and as a higher rank coal, it includes greater proportions of more recalcitrant compounds (i.e., aromatic content) that are considered to be less favorable for biogenic methane production (Fallgren et al., 2013; Strapoc et al., 2011). Thus, enrichment of

methanogenic consortia and their adaptation to higher rank coals should be beneficial for enhancing methane production when such coals act as the substrate.

Physicochemical analysis of the liquid phase was carried out on the coals used in this study. These are compiled in Table 9. Values of pH for coal samples not shown were circum-neutral (6.95 – 7.15). While it would be beneficial to conduct real-time pH measurements to have an exact view of bioreactors' behavior, an estimation for this environmental factor was obtained. The low pH (3.87) can be correlated with the low methane production when Arkansas lignite – LIG coal was used as substrate; concentrations less than 72,000 ppm (13 sft^3/ton or 0.4 scm^3/g) were measured. Significant methane generated with the Praxair Illinois #6 – PI coal type, on the other hand, suggested an environment with a possible pH (4.18) condition at which some enriched methanogenic populations could effectively function (e.g., sample ETL TSB TSB PI in Figure 5). The value of pH has an important effect on bioconversion of complex organic matter (Del Real, 2007).

Methanogens are considered the most sensitive group of microorganisms within microbial consortia, and their environmental requirements are usually prioritized. Changes in methane production and/or microbial populations can be attributed to shifts of the environmental conditions (Del Real, 2007; Zupančič and Grilc, 2012). Even though most methanogenic communities seem to be dominated by neutrophilic species with limited growth and methane production outside of the aforementioned optimal range (6.80 – 7.40) (Del Real, 2007; Franke et al., 2014; Megonigal et al., 2013), there are known methanogens that can exist in low pH environments (Ferry, 1993). Values of pH lower than 4.00 are characteristic of peat bogs, where methane production has been reported (Ferry, 1993;

Kotsyurbenko et al., 2007).

Considering that low pH can indicate that some volatile fatty acids (e.g., acetic acid, propionic acid, butyric acid) could be present in the coals before inoculation, GC-MS was used for compounds analysis. Acetic acid (pKa 4.75) was identified in aqueous extracts of Miller Black Thunder – MBT, Praxair Illinois #6 – PI and NARM Cook – NC coals, while propionic acid (pKa 4.87) was only found in the Arkansas Lignite – LIG extracts. No dissolved compounds were identified in the Deer Creek Mine Waste coal – DCWC, Deer Creek Mine Soil – DCSoil, Red Hill – RH aqueous extracts, or the other coal samples not shown. Those compounds were probably trapped in the coal structure as residual products of microbial activity due to native microorganisms and/or as residual products of the coalification process (Fallgren et al., 2013; Hayatsu et al., 1978). It is known that volatile fatty acids can be found during biodegradation of coal. They can have different and cooperative effects on bacteria and archaea (Jones et al., 2010b; Strapoc et al., 2011). Reported concentrations of acetic acid and butyric acid of 2,400 and 1,800 ppm, respectively, have not shown significant inhibitory effects on the activity of methanogens, while low concentrations of propionic acid (e.g., 900 ppm) can result in significant microbial inhibition (Franke et al., 2014; Wang et al., 2009). Consequently, the presence of propionic acid in the Arkansas Lignite – LIG aquose extracts could also be correlated to the low methane production measured during experimentation. However, quantification of the identified compounds is still required to verify this inhibitory effect.

After twenty-four weeks of incubation, methane and carbon dioxide levels dropped significantly as shown in Figure 6. Results of controls related to those samples are included in Table 10. For the sample JR2 TSB TSB DCWC a methane concentration of 4,100 ppm

(0.7 sft^3/ton or $2.2x10^{-2}$ scm^3/g) and carbon dioxide concentration of 27,312 ppm (4.8 sft^3/ton or 0.15 scm^3/g) were measured. Other microbial samples (i.e., coal and environmental samples) reached methane concentrations less than 1,100 ppm (0.19 sft^3/ton or $6x10^{-3}$ scm^3/g).

The scenario of methane content increasing and subsequently decreasing has been also reported by other researchers (Papendick et al., 2011; Zhang et al., 2015, 2016). As a possible explanation, microbial methane oxidation (anaerobic and/or aerobic) could have occurred (Megonigal et al., 2013). Headspace gas was not completely removed nor neither replaced during sampling for gas measurements; the bioreactors operated as a closed system. It is also likely that available nutrients or trace elements may have depleted over time. This depletion could have led to a decrease in the microbial populations. Finally, it is speculated that only limited amount**s** of electron donors (i.e., acetate or H_2) were available or were not continuously produced to support methanogenesis.

4.4.4. Aerotolerance

An attempt was made to assess microbial consortia functionality under initial anaerobic conditions. The microbial consortium (i.e., RH TSB TSB MBT) under argon atmosphere showed a greater methane production (255,000 ppm, 45 sft^3/ton or 1.4 scm^3/g) than the sample under initial atmospheric exposure (100,000 ppm, 18 sft^3/ton or 0.56 scm^3/g). It is shown that both microbial samples were significant methane producers (Figure 7). This suggests that the developed microbial consortia were both aerotolerant and anaerobically methanogenic, which can ease their delivering to an oxygen-free environment to digest coal and produce methane (Bum-Han et al., 2017; Zhang et al.,

2015). It is noted that the measured methane concentrations were less than the gas concentrations obtained after two weeks of incubation in the Phase III of the screening program (Figure 5). It is likely that low initial cell concentration, or changes of microbial communities may had been responsible for this behavior (Green et al., 2008).

Eventually, facultative and aerobic microorganisms present in the culture can rapidly consume oxygen, creating favorable conditions for the obligate anaerobes. Thus, a temporary air presence to the methane generating process can be handled since mentioned microorganisms are able to reduce the incoming oxygen to a low level. This feature and some intrinsic oxygen tolerance of anaerobes organisms can contribute to the coal biogasification process (Ali-Shah et al., 2014; Ibanez et al., 2007; Kato et al., 1997; Schnürer and Jarvis, 2010).

A wide range of methane yields from different coal ranks and microbial communities exposed to various strictly anaerobic enrichment and cultivation conditions has been reported (Green et al., 2008; Gupta et al., 2014; Harris et al., 2008; Jones et al., 2010; Orem et al., 2010; Papendick et al., 2011; Park and Liang, 2016; Wawrik et al., 2012). However, only a limited number of evaluations have been published where initial aerobic environments were considered.

In the study conducted by Zhang et al. (2015), for instance, formation water was collected from a coalbed methane well in southern Illinois. Those researchers developed a microbial consortium for ex situ bituminous coal bioconversion. Ground coal was obtained from the Illinois basin. During their experimentation, complete air exposure was avoided. Nitrogen gas was used to purge the bioreactors prior to incubation. Three different media were used: standard Tanner medium, mineral salt (MS) medium, and formation water was

used as control. After sixty-five days of incubation at 28°C, selected samples were added to fresh coal and an MS medium. Some of these samples were purged with nitrogen while others were in contact with the atmosphere. After twenty days of incubation, there was no statistically significant difference in the yields of methane or carbon dioxide as function of purging. The researchers suggested that an effective microbial consortium can be cultivated under conditions that are not strictly anaerobic.

Opara (2012) and Opara et al. (2012), furthermore, performed a comprehensive study of methane and carbon dioxide production. Those authors developed aerotolerant microbial consortia that generated significant quantities of methane and/or carbon dioxide in various down-selection screening of microbial and in consortia scale-up that included aerobic conditions. Bituminous coal and waste coal, lake sediments, wetland sediments, river sediments, digester sludge, as well as oil seep and gas well samples were used as sources for the microbial populations. An initial enrichment step with different growth media was carried out after collection. Gas production was evaluated using hydrocarbon materials (bituminous coal, waste bituminous coal and lignite) over a thirty-day period at 23°C. The optimal CO_2 and CH_4 producers were ultimately selected and combined into five consortia. During this experimentation, introduction of microbial populations, mainly consortia cultured from noncoal environments (i.e., not coal or waste coal), increased the rate of hydrocarbon biodegradation, with CO_2 as an end product.

Different behavior was observed in our study under similar environmental conditions with bituminous coal and using the same nutrient recipes. Samples that included consortia enriched and cultured from both hydrocarbon and environmental sources have shown significant methane production (Figure 5) after two-weeks incubation period,

suggesting their potential use to biodegrade coal and produce methane as the end product.

4.4.5. ANOVA of a Single Factor

After the three screening phases, methane and carbon dioxide concentrations at two and twenty-four weeks were separately analyzed with ANOVA single factor evaluation. Table 11 summarizes the levels that were the most or least important for methane and carbon dioxide production during the experimental program. In Table 11, a p-value lower than 0.05 means that there was a statistical difference among medians or means of each level within a defined factor with a confidence level of 95% (depending on the statistical test used). Cases where the level is not shown (p-values > 0.05) indicate that there was not a significant statistical difference. In those instances, all levels had an equal or similar impact on gas production within the evaluated factor. The factors and levels used for the variance analysis ANOVA are given in Table 4. The following trends were observed:

- Tryptic soy broth – TSB was an important nutrient medium for enriching the microbial consortia and stimulating methane production for both of the evaluated periods of incubation. On the other hand, after two weeks of incubation, lactate – LAC had a greater impact on carbon dioxide production when used as the final nutrient amendment. Yeast-urea-phosphate – YUP could be considered as a suitable initial nutrient medium**,** causing increased carbon dioxide production after twenty-four weeks of incubation.
- Acetate – ACE did not show, on average, a significant impact on gas production after two weeks or after twenty-four weeks of incubation in comparison to the other nutrient solutions used. This suggests that the microbes could had been

inhibited or possibly required more complex nutrients than the acetate in order to be enriched and positively stimulated.

- NaCl - 8.5 g/L - showed a negative impact on methane production. This was expected since NaCl was used in the control samples. NaCl was included in the statistical analysis since it was associated with samples that were chosen based on their physical appearance. As mentioned earlier, nutrient amendments with specific ingredients were needed to satisfactorily stimulate microbial growth, and eventually to promote gas production.
- The coal type itself is relevant. Bituminous Deer Creek Mine Waste coal – DCWC had the greatest positive impact on methane production in both periods of incubation (i.e., two weeks and twenty-four weeks). Reduced inhibition of ex situ cultured microbial populations could have been operative. This probably made this coal the most suitable sample for consortia adaptation and subsequent methane generation. At the other extreme, Arkansas Lignite – LIG had a negative impact on methane production. As was mentioned earlier, the low pH of the liquid media associated with this coal could be correlated with the low methane concentrations, inhibiting methanogenesis to some extent.
- The initial source of the microbial community, not surprisingly, is also relevant. The Jordan River 2 – JR2 sediment sample was an effective source of microbes for methane production at two weeks of incubation, but not at twenty-four weeks. This most likely indicates that active methanogenic populations were positively stimulated and initially generated significant amounts of gas. However, there were limited electron donors (i.e., acetate or H_2) available,

and/or they were not continuously produced. The East Texas Lignite – ETL was an important source of microbes for methane production at twenty-four weeks in Phase III screening. The Deer Creek Mine Soil – DCSoil afforded enhanced carbon dioxide production after twenty-four weeks of incubation.

4.5. Summary and Implications

This study demonstrated that methane and carbon dioxide generating microbial communities from coal and lake sediments can be sequentially enriched and adapted through a matrix of screening/high grading steps. Consortia can be developed that could have commercial viability. Development of methanogenic microbial consortia under incomplete anaerobic conditions makes these consortia appropriate and low cost biological complements for increasing methane productivity.

These consortia should tolerate oxygen exposure during culturing, storage and injection into a target coalbed (in situ application). For ex situ applications, these microbial consortia could be high graded for use on coal waste heaps or bioreactors where oxygen exposure is anticipated. Converse et al. (2001) and Clement et al. (2012) describe using methanogens for in situ and ex situ scenarios.

High methane concentrations obtained during the last phase of the screening indicated that active methanogenic populations were cultured with a suitable combination of nutrient media and coal sources. Small scale pilot testing would be a rational next step, starting with ex situ demonstrations. In addition, identification of microbial populations present in promising samples would reveal the possible presence of known or novel methanogenic species, which will be subsequently taken into consideration for further

studies.

The hydrocarbon samples evaluated in this study, such as oil shale and waxy crude were not effective sources of appropriate methanogenic populations.

As is well known, identification of appropriate nutrient amendments for methanogenic communities is an important element for developing microbial consortia. This reduces the microbial screening requirements, and provides possibilities for evaluating cost-effective and optimized coal biodegradation and methane production.

Table 2. Proximate analysis, rank, and location of coal samples

Provenance	Moisture[a] wt.%	Ash[b] wt. %	Volatile [b] wt.%	Fixed carbon[b] wt.%	Heating value[b] Btu/lb	Heating value[c] Btu/lb	Coal rank	Location
Miller Black Thunder - MBT	10.21	6.56	48.23	45.21	11077	11855	Subbituminous	Powder River Basin, WY
NARM Cook - NC	11.31	6.02	47.32	46.66	10821	11514	Subbituminous	North Antelope Mine, WY
North River - NR	1.58	13.26	35.13	51.61	12811	14770	Bituminous	Alabama, AL
Praxair Illinois #6 - PI	3.36	10.04	39.11	50.85	12195	13556	High volatile bituminous	Illinois, IL
Red Hills - RH	11.70	24.02	43.74	32.24	8776	11537	Lignite	AcKerman, MI
Utah Skyline - US	2.98	8.85	52.79	38.36	12816	14060	Bituminous	Utah Skyline Mine, UT
Arkansas Lignite - LIG	11.10	9.36	58.18	32.46	11294	12460	Lignite	Arkansas, AR
Waste Coal - DCWC	1.56	5.04	47.21	47.75	14690	15470	Bituminous	Deer Creek Mine, UT
Soil - DCSoil	1.69	51.12	28.52	20.36	6222	12729	NA	Deer Creek Mine, UT
East Texas - ETL	5.01	25.86	49.37	24.77	8790	11856	Lignite	East Texas, TX

Table 3. Ultimate analysis

Provenance	Hydrogen[b] wt.%	Carbon[b] wt.%	Nitrogen[b] wt.%	Sulfur[b] wt.%	Oxygen[b] wt.%
Miller Black Thunder - MBT	4.32	67.24	0.99	0.36	20.53
NARM Cook - NC	4.35	67.71	0.98	0.28	20.66
North River - NR	4.75	70.45	1.71	2.08	7.74
Praxair Illinois #6 - PI	4.54	67.15	1.34	3.59	13.34
Red Hills - RH	3.72	51.56	1.09	0.85	18.76
Utah Skyline - US	5.11	71.12	1.50	0.56	12.87
Arkansas Lignite - LIG	5.39	63.34	0.88	1.20	19.83
Waste Coal - DCWC	5.60	74.63	1.46	0.44	12.84
Soil - DCSoil	5.46	36.53	0.73	0.52	5.64
East Texas - ETL	3.86	51.80	1.03	0.98	16.47

[a] As-received, [b] Dry basis, [c] Moisture, and ash free

Table 4. Factors and levels for variance analysis ANOVA

Factor	Level
Sources of microbial community	Deer Creek Mine Soil - DCSoil; Deer Creek Mine Waste coal - DCWC; Jordan River - JR Samples 1 and 2; East Texas Lignite - ETL; Miller Black Thunder - MBT; NARM Cook - NC; North River - NR; Praxair Illinois #6 - PI; Red Hills - RH; Utah Lake - UL Samples 4, 5 and 6; Utah Skyline - US.
Initial nutrient medium	Acetate - ACE; tryptic soy broth - TSB; lactate - LAC; acetate - yeast-phosphate medium - AYP; yeast-urea-phosphate medium - YUP; sodium chloride - NaCl.
Coal type	Deer Creek Mine Waste coal - DCWC; Miller Black Thunder - MBT; Praxair Illinois #6 - PI; Arkansas Lignite - LIG
Final nutrient medium	Acetate - ACE; tryptic soy broth - TSB; lactate - LAC

Table 5. Results of controls related to samples in Figure 3

Control samples		DCWC	ETL	RH	UL6	UL4	JR1	UL5
CH_4	ppm	7.33	256	6.66	4.42	5.67	71.32	5.67
	sft^3/ton	$1.29x10^{-3}$	$4.51x10^{-2}$	$1.17x10^{-3}$	$7.79x10^{-4}$	$9.99x10^{-4}$	$1.26x10^{-2}$	$9.99x10^{-4}$
CO_2	ppm	2,354	13,566	22,058	6,906	2,941	4,907	2,941
	sft^3/ton	$4.15x10^{-1}$	2.39	3.89	1.22	$5.18x10^{-1}$	$8.65x10^{-1}$	$5.18x10^{-1}$
Control samples		**DCSoil**	**JR2**	**US**	**MBT**	**NR**	**NC**	**PI**
CH_4	ppm	3.18	3.32	0.27	7.65	78.72	6.85	20.08
	sft^3/ton	$5.60x10^{-4}$	$5.85x10^{-4}$	$4.76x10^{-5}$	$1.35x10^{-3}$	$1.39x10^{-2}$	$1.21x10^{-3}$	$3.54x10^{-3}$
CO_2	ppm	4,028	3,517	632	53,271	20,006	33,966	32,567
	sft^3/ton	$7.10x10^{-1}$	$6.20x10^{-1}$	$1.12x10^{-1}$	9.39	3.53	5.99	5.74

Table 6. Results from added nutrients in Phase I screening

Nutrient amendments		TSB	LAC	ACE	YUP	AYP
CH_4	ppm	12,600	142,200	44,560	48,745	59,195
CO_2	ppm	8,420	158,720	57,297	40,390	63,163

Table 7. Results from added nutrients

Nutrient amendments		TSB	LAC	ACE
CH_4	ppm	8,400	105,650	30,750
CO_2	ppm	5,610	117,915	39,540

Table 8. Results of controls related to samples in Figure 5

Control samples		DCWC-TSB	DCWC-LAC	MBT-TSB	MBT-LAC	PI-TSB	PI-LAC
CH_4	ppm	8.31	5.37	5.80	8.21	13.81	17.02
	sft^3/ton	$1.46x10^{-3}$	$9.46x10^{-4}$	$1.02x10^{-3}$	$1.44x10^{-3}$	$2.43x10^{-3}$	$2.99x10^{-3}$
CO_2	ppm	114,451	103,195	157,348	43,247	78,980	83,407
	sft^3/ton	20.17	18.19	27.73	7.62	13.92	14.70

Table 9. pH of liquid phase in coals tested

Designation	Coal samples	pH
LIG	Arkansas Lignite	3.87 ± 0.06
PI	Praxair Illinois #6	4.18 ± 0.01
NC	NARM Cook	6.68 ± 0.02
MBT	Miller Black Thunder	6.78 ± 0.01
DCWC	Deer Creek Mine Waste coal	7.06 ± 0.02

Table 10. Results of controls related to samples in Figure 6

Control samples		DCWC-TSB	DCWC-LAC	MBT-TSB	MBT-LAC	PI-TSB	PI-LAC
CH_4	ppm	8.33	5.26	5.84	8.06	13.78	17.47
	sft^3/ton	$1.47x10^{-3}$	$9.26x10^{-4}$	$1.03x10^{-3}$	$1.42x10^{-3}$	$2.43x10^{-3}$	$3.08x10^{-3}$
CO_2	ppm	114,081	102,919	157,040	43,003	103,384	107,440
	sft^3/ton	20.11	18.13	27.67	7.57	18.22	18.94

Table 11. Main controls during microbial consortia creation

	Factor	Statistical Test	p-value	Average Rank for Level of Highest Impact	Level of Highest Impact	Average Rank for Level of Lowest Impact	Level of Lowest Impact
Methane concentration at two weeks	SM	Kruskal-Wallis	0.0	212.2	JR2	61.7	PI
	INM	Kruskal-Wallis	4.5×10^{-9}	167.4	TSB	48.1	NaCl
	CT	Kruskal-Wallis	2.7×10^{-8}	176.6	DCWC	95.8	LIG
	FNM	Kruskal-Wallis	2.6×10^{-8}	170.2	TSB	103.9	ACE
Methane concentration at twenty-four weeks	SM	Kruskal-Wallis	5.9×10^{-5}	189.8	ETL	97.7	JR2
	INM	Kruskal-Wallis	4.7×10^{-2}	156.6	TSB	95.3	NaCl
	CT	Kruskal-Wallis	1.2×10^{-2}	165.3	DCWC	119.9	LIG
	FNM	Kruskal-Wallis	3.1×10^{-5}	172.2	TSB	117.4	ACE
Carbon dioxide concentration at two weeks	SM	Kruskal-Wallis	1.7×10^{-1}	-	-	-	-
	INM	Anova	1.5×10^{-1}	-	-	-	-
	CT	Kruskal-Wallis	5.6×10^{-11}	177.9	MBT	87.0	DCWC
	FNM	Anova	0.0	-	LAC	-	ACE
Carbon dioxide concentration at twenty-four weeks	SM	Anova	2.4×10^{-3}	-	DCSoil	-	DCWC
	INM	Kruskal-Wallis	9.1×10^{-3}	202.1	YUP	133.5	LAC
	CT	Anova	1.6×10^{-1}	-	-	-	-
	FNM	Kruskal-Wallis	2.9×10^{-6}	177.7	TSB	118.3	ACE

SM = Source of microbial community, INM = initial nutrient medium, CT = coal type, FNM = final nutrient medium

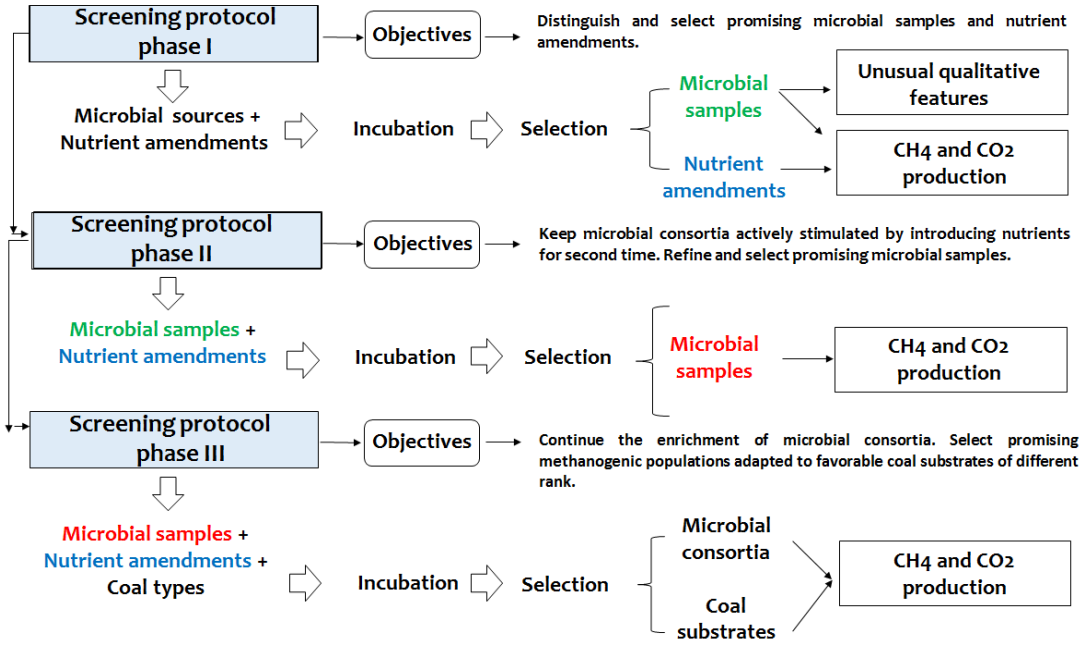


Figure 2. Diagram of screening protocols

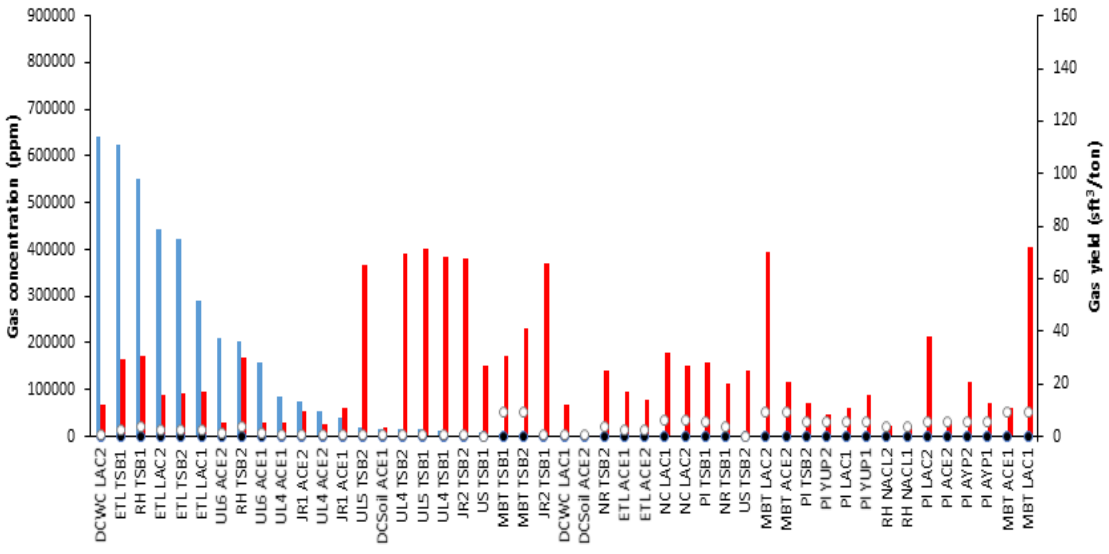


Figure 3. Methane (blue bars) and carbon dioxide (red bars) concentrations from Phase I screening. The black and white points represent methane and carbon dioxide concentrations from controls samples, respectively.

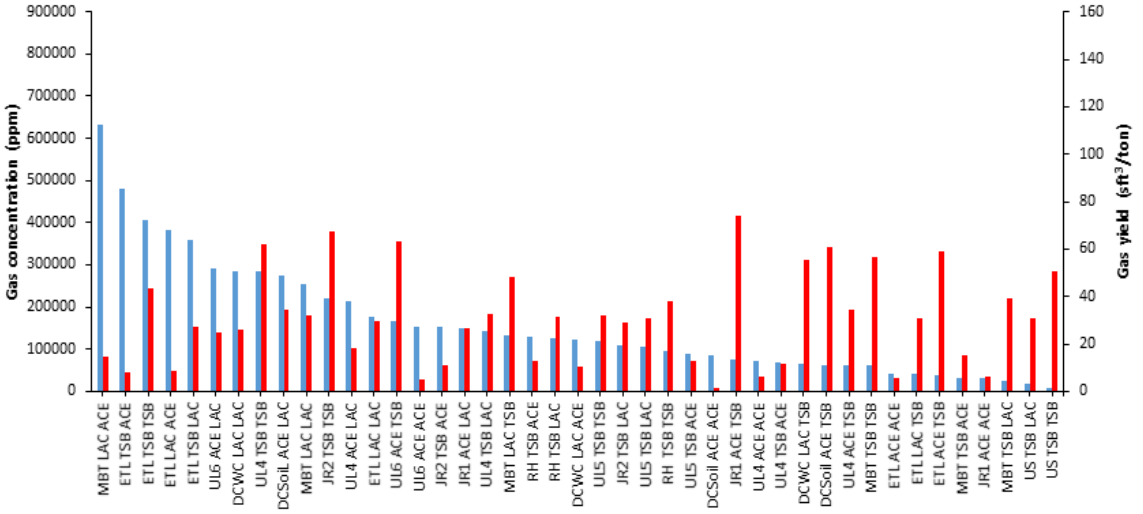


Figure 4. Methane (blue bars) and carbon dioxide (red bars) concentrations from Phase II screening.

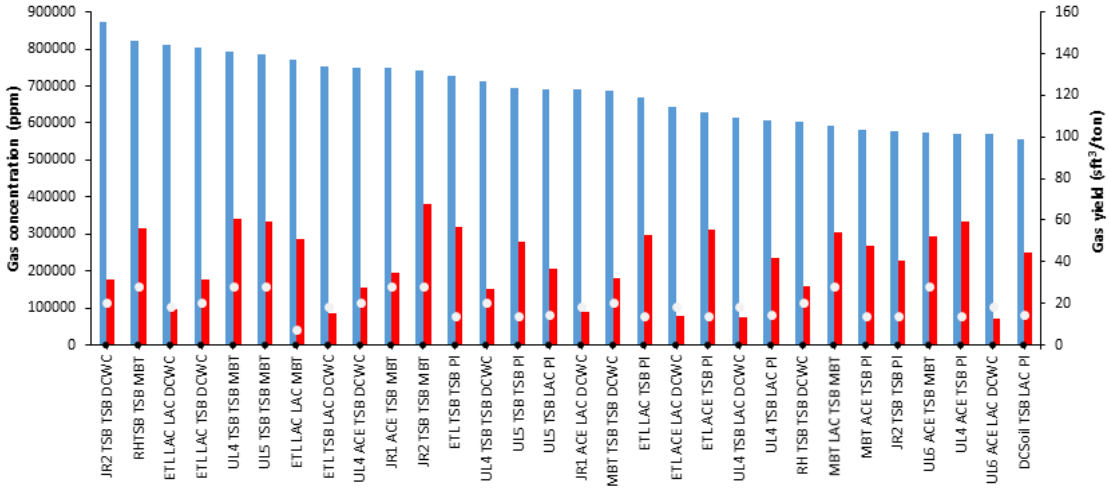


Figure 5. Methane (blue bars) and carbon dioxide (red bars) concentrations generated during the Phase III screening after two weeks. The black and white points represent methane and carbon dioxide concentrations from control samples, respectively.

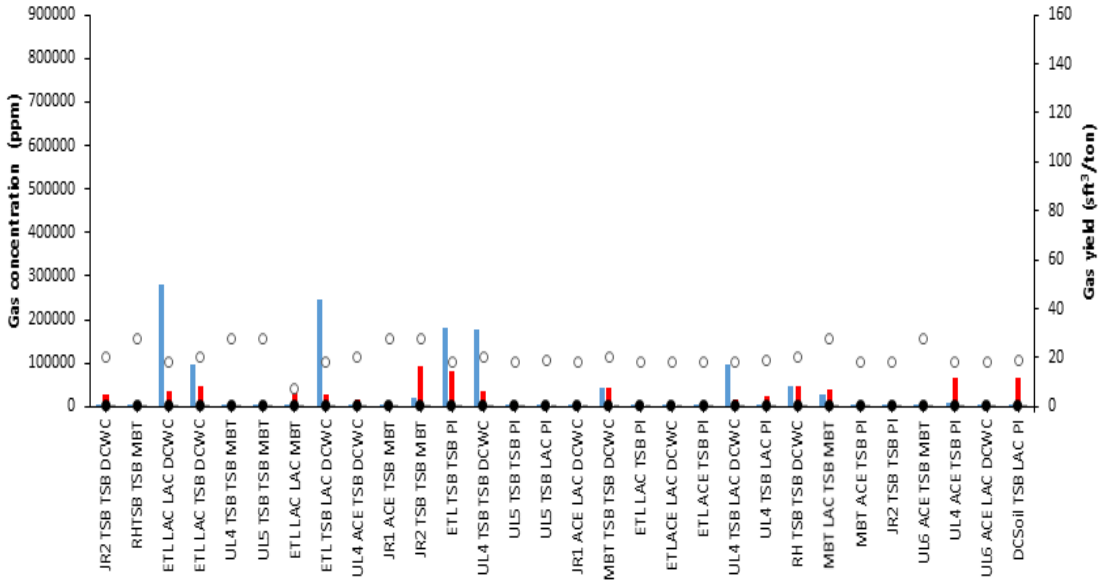


Figure 6. Methane (blue bars) and carbon dioxide (red bars) concentrations generated during the Phase III screening after twenty-four weeks. The black and white points represent methane and carbon dioxide concentrations from control samples, respectively.

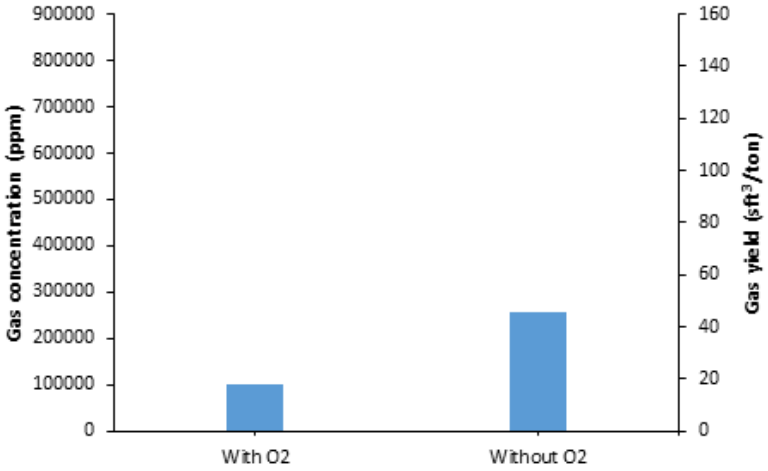


Figure 7. Methane production under initial atmospheric exposure (with oxygen) and initial anaerobic conditions (without oxygen).

4.6. References

Ali-Shah, F., Mahmood, Q., Maroof-Shah, M., Pervez, A., Ahmad-Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Scientific World J. Art. No. 183752. http://doi.org/10.1155/2014/183752.

ASTM E515-05, 2005. Standard Test Method for Leaks Using Bubble Emission Techniques. ASTM International, West Conshohocken, PA. doi:10.1520/E0515-05.

Bao, Y., Huang, H., He, D., Ju, Y., Qi, Y., 2016. Microbial enhancing coal-bed methane generation potential, constraints and mechanism – a mini-review. J. Nat. Gas Sci. Eng. 35, 68-78. https://doi.org/10.1016/j.jngse.2016.08.035.

Bum-Han, K., Fuertez, J., Stella, A., Nguyen, V., Szendrei, A., Mclennan, J.D., Sparks, T.D., 2017. Calcium alginate polymer encapsulation of proppant with time-release delivery of microbial consortia for methanogenesis. Energy Fuels. Submitted.

Buswell, E.G., Neave, S.L., 1930. Laboratory studies of sludge digestion. Illinois Division of State Water Survey, Bulletin No. 30. http://hdl.handle.net/2142/94553. (accessed 16.10.2015).

Converse, D., Hinton, S., Hieshima, G., Barnum, R., Mahankumar, S., 2001. Process for stimulating microbial activity in a hydrocarbon-bearing, subterranean formation. U.S. Patent Application 20010045279 A1.

Clement, B.G., Ferry, J.G., Underwood, S., 2012. Methods to stimulate biogenic methane production from hydrocarbon-bearing formations. U.S. Patent Application 20120138290 A1.

Del Real, J.O., 2007. Evaluation and modeling of anaerobic depuration kinetics from stillage of alcohol industry. Ph.D. Dissertation. Institute of Basic Sciences and Engineering, Autonomous University of Hidalgo State. Mexico.

Faiz, M., Hendry, P., 2006. Significance of microbial activity in Australian coal bed methane reservoirs – a review. Bull. Can. Pet. Geol. 54 (3), 261-272. doi:10.2113 /gscpgbull.54.3.261.

Fallgren, P., Jin, S., Zeng, C., Ren, Z., Lu, A., Ren, S., 2013. Comparison of coal rank for enhanced natural gas production. Int. J. Coal Geol. 115, 92-96. https://doi.org/ 10.1016/j.coal.2013.01.014.

Ferry J.G., 1993. Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall, New York.

Franke, I.H., Walter, A., Ebner, C., Insam, H., 2014. Investigation into the effect of high

concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag. 34 (11), 2080-2089. https://doi.org/10.1016/j.wasman 2014.07.020.

Green, M.S., Flanegan, K.C., Gilcrease, P.C., 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76, 34-45. https://doi.org/10.1016/j.coal.2008.05.001.

Gupta, P., Gupta, A., 2014. Biogas production from coal via anaerobic fermentation. Fuel. 118, 238-242. https://doi.org/10.1016/j.fuel.2013.10.075.

Harris, S.H., Smith, R.L., Barker, Ch.E., 2008. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. Int. J. Coal Geol. 76, 46-51. https://doi.org/10.1016/j.coal.2008.05.019.

Head, I.M., Gray, N.D., Larter, S.R., 2014. Life in the slow lane: Biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front. Microbiol. 5, 1-23. doi:10.3389/fmicb.2014.0056 6.

Hecht, E., Lighty, J., Shaddix, C., 2013. Kinetic rates of oxidation and gasification reaction of coal chars reacting in oxy-combustion environments, in: 8th U.S National Combustion Meeting. Park City, Utah. U.S. May 19th-22nd. http://combustion 2013. utah.edu/opencms/opencms/home (accessed 22.10.2016).

Ibanez, J.G., Hernandez-Esparza, M., Doria-Serrano, C., Fregoso-Infante, A., Mohan, M., 2007. Environmental Chemistry: Fundamentals. Springer Science-Business Media, New York.

Jones, E., Voytek, M., Corum, M., Orem W., 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76, 7013-7022. doi:10.1128/AEM.00728-10.

Kato, M.T., Field, J.A., Lettinga, G., 1997. Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. Braz. J. Chem. Eng. 14 (4), 1678-4383. http://dx.doi.org/10.1590/S0104-66321997000400015.

Kayhanian, M., Tchobanoglous, G., Brown, R., 2007. Biomass conversion processes for energy recovery, in: Kreith, F., Goswami, D.G. (Eds.), Handbook of Energy Conservation and Renewable Energy. Taylor and Francis Group., Londres, pp. 67. https://doi.org/10.1201/9781420003482.ch25.

Kotsyurbenko, O.R., Friedrich, M.W., Simankova, M.V., Nozhevnikova, A.N., 2007. Shift from acetoclastic to H_2-dependent methanogenesis in a West Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacerium* strain. Appl. Environ. Microbiol. 73 (7), 2344-2348. doi:10.1128/AEM.02413-06.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. J. Am. Stat. Assoc. 47 (260), 583- 621. http://www.jstor.org/stable/2280779.

Lema, J.M., Mendez, R., Iza, J., García, P., Fernandez-Polanco, F., 1991. Chemical reactor engineering concepts in design and operation of anaerobic treatment processes. Wat. Sci. Tech. 24 (8), 79-86. http://wst.iwaponline.com/content/24/8/79.

Lizasoain, L., Joaristi, L., 2003. Management and Data Analysis with SPSS. S.A Editions Paraninfo, Madrid.

Li, X., Fu, X., Liu, A., An, H., Wang, G., Yang, X., Wang, L., Wang, H., 2016. Methane adsorption characteristics and adsorbed gas content of low-rank coal in China. Energy Fuels. 30 (5), 3840-3848. doi:10.1021/acs.energyfuels.6b00071.

Liu, Y., Boone, D.R., 1991. Effects of salinity on methanogenic decomposition. Bioresour. Technol. 35 (3), 271-273. https://doi.org/10.1016/0960-8524(91)90124-3.

Megonigal, J.P., Hines, M.E., Visscher, P.T., 2013. Anaerobic metabolism: Linkages to trace gases and aerobic processes, in: Turekian, K., Holland, H. (Eds.), Treatise on Geochemistry, second ed. Elsevier Inc., Oxford, pp. 273-359. doi:10.1016/B978-0-08-095975-7.00808-1.

Menger, W.M., Kern, E.E., Karkalits, O., Wise, D.L., Leuschner, A.P., Odelson, D., 2000. Microbial process for producing methane form coal. U.S. Patent Application. 6143534A.

Meslé, M., Dromart, G., Oger, P., 2013. Microbial methanogenesis in subsurface oil and coal. Res. Microbiol. 164 (9), 959-972. https://doi.org/10.1016/j.resmic.2013.07.004.

Naftz, D.L., Millero, F.J., Jones, B.F., Green, W.R., 2011. An equation of state for hypersaline water in Great Salt Lake, Utah, U.S. Aquat. Geochem. 17 (6), 809-820. doi:10.1007/s10498-011-9138-z.

Opara, A., Adams, D.J., Free, M.L., Mclennan, J., Hamilton, J., 2012. Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste materials. Int. J. Coal Geol. 96–97, 1-8. https://doi.org/10.1016/j.coal.2012.02.010.

Opara, A., 2012. Biochemically enhanced methane production from coal. PhD. Dissertation. Department of Civil and Environmental Engineering, University of Utah. U.S.

Orem, W.H., Voytek, M.A., Jones, E.J., Lerch, H.E., Bates, A.L., Corum, M.D., 2010. Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. Org. Geochem. 41 (9), 997-1000. https://doi.org/10.1016/j.orggeochem.2010.03.005.

Papendick, S.L., Downs, K.R., Vo, K.D., Hamilton, S.K., Dawson, G.K., Golding, S.D., 2011. Biogenic methane potential for Surat Basin, Queensland coal seams. Int. J. Coal Geol. 88, 123-134. https://doi.org/10.1016/j.coal.2011.09.005.

Park, Y.S., Liang, Y., 2016. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). Fuel. 166, 258-267. https://doi.org/10.1016/j.fuel.2015.10.121.

Patel, G.B., Roth, L.A., 1977. Effect of sodium chloride on growth and methane production of methanogens. Can. J. Microbiol. 23 (7), 893-897. doi:10.1139/m77-131.

Pfeiffer, R.S., Ulrich, G.A., DeBruyn, R.P., Vanzin, G., 2011. Chemical amendments for the stimulation of biogenic gas generation in deposits of carbonaceous material. U.S. Patent Application 7977282 B2.

Prior, W., Clardy, B., Baber, Q., 1985. Arkansas geological commission. Information Circular 28-C. http://www.geology.ar.gov/infocirculars/ ic28c.htm (accessed 10.5. 2015).

Rathi, R., Priya, A., Vohra, M., Lavania, M., Lal, B., Sarma, P., 2015. Development of a microbial process for methane generation from bituminous coal at thermophilic conditions. Int. J. Coal Geol. 147-148, 25-34. https://doi.org/10.1016/j.coal.2015. 06.004.

Ritter, D., Vinson, D., Barnhart, E., Akob, D., Fields, M., Cunningham, A., Orem, W., McIntosh, J., 2015. Enhanced microbial coalbed methane generation: A review of research, commercial activity, and remaining challenges. Int. J. Coal Geol. 146, 28-41. https://doi.org/10.1016/j.coal.2015.04.013.

Sarv, H., Chen, Z., Sayre, A., Maringo, G., 2009. Oxygen-enriched combustion of a Powder River basin black thunder coal for NOx reduction in a cyclone furnace, in: The 34th International Technical Conference on Clean Coal and Fuel Systems. Florida, U.S. May 31st-Jun 4th. http://www.proceedings.com/08499.html (accessed 11.5.2015).

Scott, A.R., Guyer, J.E., 2004. Method of generating and recovering gas from subsurface formations of coal, carbonaceous shale and organic-rich shales. U.S. Patent Application 20040033557 A1.

Schnürer, A., Jarvis, A., 2010. Microbiological handbook for biogas plants. Swedish Waste Management U2009:03. Swedish Gas Centre Report 207. http://www.eac-quality.net/fileadmin/eac_quality/user_documents/3_pdf/Microbiological_handbook_for_biogas_plants.pdf (accessed 15.2.2016).

Schink, B., 2006. Syntrophic associations in methanogenic degradation. Prog. Mol. Subcell. Biol. 41, 1-19. doi:10.1007/3-540-28221-1_1.

Senthamaraikkannan, G., Budwill, K., Gates, I., Mitra, S.K., Prasa, V., 2016. Kinetic modeling of the biogenic production of coalbed methane. Energy Fuels. 30 (2), 871-883. doi:10.1021/acs.energyfuels.5b02450.

Shuler, M.L., Kargi, F., 2002. Bioprocess Engineering: Basic Concepts, second ed. Prentice Hall PTR, New Jersey.

Smith, I.M., 2010. Microbial methane from carbon dioxide in coal beds. IEA Clean Coal Center. ISBN 978-92-9029-494-8, https://www.usea.org/publication/ microbial-methane-carbon-dioxide-coal-beds-ccc174 (accessed 10.11.2015).

Speight, J.G., 2005. Handbook of Coal Analysis. John Wiley and Sons Inc, New Jersey.

Speight, J.G., 1983. The Chemistry and Technology of Coal. Marcel Dekker Inc, New York.

Srivastava, K.C., Walia, D.S., 1997. Biological production of humidic acid and clean fuels form coal. U.S. Patent. 5670345 A.

Strapoc, D., Mastalerz, M., Dawson. K., Macalady, J., Callaghan, A., Wawrik, B., Turich, C., Ashby, M., 2011. Biogeochemistry of microbial coalbed methane. Annu. Rev. Earth Planet. Sci. 39, 617-656. https://doi.org/10.1146/annurev-earth-040610-133343.

Sturm, P.A., 1980. The Great Salt Lake brine system, in: Gwynn J.W. (Eds.), Great Salt Lake: A Scientific, Historical, and Economic Overview. Utah Geological and Mineral Survey., Salt Lake City, pp. 147-162. http://digitallibrary.utah.gov/awweb/awarchive?item=39078.

Ulrich, G., Bower, S., 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. Int. J. Coal Geol. 76, 25-33. https://doi.org/10.1016/j.coal.2008.03.006.

Vaysman, V., Yixin, L., 2012. Detailed coal specifications. Quality guidelines for energy system studies. National Energy Technology Laboratory. U.S. Final Report. DOE/NETL-401/012111. https://www.netl.doe.gov/File%20Library/research/energy%20analysis/publications/QGESS_DetailCoalSpecs_Rev4_20130510.pdf (accessed 5.2.2015).

Wagner, A.O., Hohlbrugger, P., Lins, P., Illmer, P., 2012. Effects of different nitrogen sources on the biogas production – a lab-scale investigation. Microbiol. Res. 167, 630-636. doi:10.1016/j.micres.2011.11.007.

Wang, B., Tai, C., Wu, L., Chen, L., Liu, J., Hu, B., Song, D., 2017. Methane production from lignite through the combined effects of exogenous aerobic and anaerobic microflora. Int. J. Coal Geol. 173, 84-93. https://doi.org/10.1016/j.coal.2017.02.

012.

Wawrik, B., Mendivelso, M., Parisi, V.A., Suflita, J.M., Davidova, I.A., Marks, C.R., 2012. Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. FEMS Microbiol. Ecol. 81, 26-42. doi:10.1111/j.1574-6941.2011.01272.x.

White, C., Smith, D.H., Jones, K.L., Goodman, A.L., Jikich, S.A., Lacount, R.B., Dubose, S.B., Ozdemir, E., Morsi, B.I., Schroeder, K.T., 2005. Sequestration of carbon dioxide in coal with enhanced coalbed methane recovery – a review. Energy Fuel. 19 (3), 659-724. doi:10.1021/ef040047w.

Wise, D., 1990. Bioprocessing and Biotreatment of Coal. Marcel Dekker Inc, New York.

Xiao, D., Peng, S., Wang, B., Yan, X., 2013. Anthracite bio-degradation by methanogenic consortia in Qinshui Basin. Int. J. Coal Geol. 116-117, 46-52. https://doi.org/10.1016/j.coal.2013.06.008.

Zhang, J., Liang, Y., Pandey, R., Harpalani, S., 2015. Characterizing microbial communities dedicated for conversion of coal to methane in situ and ex situ. Int. J. Coal Geol.146, 145-154. https://doi.org/10.1016/j.coal.2015.05.001.

Zhang, J., Park, S., Liang, Y., Harpalani, S., 2016. Finding cost-effective nutrient solutions and evaluating environmental conditions for biogasifying bituminous coal to methane ex situ. Appl. Energy. 165, 559-568. https://doi.org/10.1016/j.apenergy.2015.12.067.

Zupančič, G.D., Grilc, V., 2012. Anaerobic treatment and biogas production from organic waste, in: Kumar, S., Bharti, A. (Eds.), Management of Organic Waste. InTech., Rijeka, pp. 1-28. doi:10.5772/32756.

5. POTENTIAL APPLICATION OF METHANOGENIC MICROBIAL CONSORTIA FOR COAL BIOGASIFICATION

5.1. Abstract

Biogasification of coal or microbially enhanced coalbed methane has become an important research topic recently. The biological conversion of coal to methane can be conceived as a feasible and environmental friendly approach for improving coalbed methane production. Within the strategies for stimulation of gas production, the addition of a microbial consortium or bioaugmentation can be seen as a promising alternative. However, relatively few studies have been conducted on the strategies for enriching microbial population ex situ under initial atmospheric exposure for subsequent injection into coal seams to stimulate biodegradation and methanogenesis. The development of methanogenic microbial consortia, especially those that can tolerate moderate and low oxygen concentrations and still retain anaerobic functionality, could be as an attractive biological complement for coal biogasification. Promising microbial consortia were incubated using low concentrations of nutrient amendments (e.g., 22.4% v/v, 3.36 mg/cm^3 TSB) and [NaCl] 6.6 mg/cm^3 as a possible scenario and foresee the elevated costs of nutrient utilization at large-scale operations (i.e., in situ and/or ex situ applications). Incubation periods of up to four months were evaluated at 23°C. Headspace concentrations of CH_4 and CO_2 were analyzed by gas chromatography. After sixty-one days of incubation for the most promising microbes, generated headspace gas concentrations reached 95,700

ppm (14 sft^3/ton) for methane and 37,560 ppm (5.5 sft^3/ton) for carbon dioxide. Microbial diversity in promising consortia was investigated. Both bacteria and archaea were identified.

5.2. Introduction

Research in the area of coal biogasification or microbially enhanced coalbed methane has gained more attention in the last few years (Bao et al., 2016; Zhang et al., 2016). As a potential energy resource and with an already established recovery, transportation and processing infrastructure, biogenic gas production can be seen as an important supplement to conventional methane extraction from coalbeds. Among the known techniques for microbially enhanced coalbed methane production, microbial augmentation or bioaugmentation (i.e., adding new or additional microorganisms to the coal in order to enhance or initiate microbial CBM production) emerges as a promising alternative. However, multiple challenges are conceived whenever this strategy is considered (Park and Liang, 2016). There remains uncertainly about the implementation of microbial consortia, their effectiveness, their sustainability, and environment constraints; beyond significant knowledge gaps related to performance and effective operations (Ritter et al., 2015).

Considering the implications and emphasizing the adverse conditions that methanogenic microbes might need to face during their implementation, important opportunities were identified for developing carbon-degrading microbial consortia, especially those that can tolerate initial atmospheric exposure and still retain anaerobic functionality (Fuertez et al., 2017; Opara, 2012; Opara et al., 2012). Methanogens are

commonly known as strict anaerobes (Wolfe, 2011), which makes it challenging to consider the application of strict anaerobic consortia without losing their activity during commercial operations.

Strict anaerobic conditions have been suggested to culture methanogenic microbial communities on coal (Furmann, 2011; Furmann et al., 2012; Green et al., 2008; Gupta and Gupta, 2014; Harris et al., 2008; Jones et al., 2010; Orem et al., 2010; Papendick et al., 2011; Rathi et al., 2015; Wawrik et al., 2012; Wolfe, 2011). There has been relatively less research related to the development of methanogenic microbial consortia that, after being exposed to oxygen environments, exhibit methanogenic activity. From a practical perspective, for large scale applications for both in situ and ex situ operations, these aerotolerant microbial communities should be seriously considered (Fuertez et al., 2017; Opara, 2012; Opara et al., 2012; Zhang et al., 2015).

As shown in Fuertez et al. (2017), microbial populations of natural consortia can be collected from various hydrocarbon-rich environments and locations characterized by natural methanogenesis to develop microbial populations able to convert select coal material into methane during initial atmospheric exposure. Through three screening phases, favorable samples can be high graded after a continuous enrichment with fresh nutrient amendments and a final adaptation stage to selected coal types. Reported headspace gas concentrations have reached 873,400 ppm (154 sft^3/ton) for methane and 176,370 ppm (31 sft^3/ton) for carbon dioxide, indicating that active methanogenic populations can be cultured with suitable combinations of nutrient media and coal sources (Fuertez et al., 2017).

As part of the aforementioned study, the potential application of the developed

methanogenic microbial consortia for coal biogasification under initial atmospheric exposure is examined. Promising microbial consortia were incubated at low concentration of nutrient amendments and salt presence as a possible scenario and foresee the elevated costs of nutrient utilization at large-scale operations (i.e., in situ and/or ex situ applications).

Methane and carbon dioxide concentrations were monitored over time using gas chromatography to evaluate microbial consortia ability to maintain gas production. Results of this study are expected to encourage the application of bioaugmentation strategy, as well to lead to in-depth research on development and utilization of methanogenic consortia under incomplete anaerobic conditions.

5.3. Materials and Methods

5.3.1. Experimental Setup

Aseptic techniques were used to conduct experimentation in this study. These included sterilization of tools and solutions (i.e., nutrient amendments, salt solution) in an autoclave (121°C, 25 min), use of gloves in collecting, and handling of microbial samples, disinfection of sample station (i.e., microbiology hood) with 10% Chlorox solution before and after work, and turning UV light under the hood for 30 min prior use.

All microbial samples were kept at a nominal temperature of 23°C. Sterile, 50-mL centrifuge tubes were used as bioreactors (Adams and Opara, 2015; Fuertez et al., 2017). These were set aside without agitation over the desired reaction periods. Two-hundred microliters (200 µL) of produced gas were directly extracted under sterile conditions using a gas-tight syringe (Hamilton Co., GASTIGHT® #1750) through a small hole in the caps

that were completely covered with silicone gel. Silicone was periodically reapplied to the end caps to prevent leakage (Bao et al., 2016; Fallgren et al., 2013). Methane and carbon dioxide production were monitored in the headspace of the bioreactors using gas chromatography.

During experimentation, the headspace of the bioreactors was not initially sparged with nitrogen gas. This allowed the cultured microbes to evolve under initial atmospheric exposure that would be similar to those conditions at which they were recollected and cultured during their development (Fuertez et al., 2017). The amounts of microbial inoculum, coal, nutrient medium, and salt solution were chosen so that about one third of the total volume corresponded to headspace. All enriched consortia were exposed to the atmosphere during cultivation and subsequent steps. These initial aerobic conditions were intentionally chosen to develop the experimental program described in this study. This demonstrated the ability for microbial consortia to survive and produce methane at low to moderate oxygen concentrations. An important consideration for their use in field-scale operations since maintaining strict anaerobic conditions would be difficult and expensive.

5.3.2. Coal Sources and Nutrient Media

The coal samples and nutrient solutions used in the current study were the same type as what were investigated and reported before. Additionally, samples were labeled according to our previous work (Fuertez et al., 2017). Two coal substrates were used herein since the selected microbial samples were exclusively adapted to these coal types: bituminous Deer Creek Mine Waste coal – DCWC and subbituminous Miller Black Thunder coal – MBT. These coals were provided in pulverized form (< 0.42 mm particle

size) by the Industrial Combustion and Gasification Research Facility at the University of Utah. The bituminous coal (dry weight basis) contained 74.63% of carbon, 1.46% of nitrogen, 5.60% of hydrogen, 0.44% of sulfur, and 12.84% of oxygen. The proximate analysis revealed that this coal had 5.04%, 47.21%, and 47.75% of ash, volatile matter and fixed carbon, respectively. The subbituminous coal, on the other hand, contained (dry weight basis) 67.24% of carbon, 0.99% of nitrogen, 4.32% of hydrogen, 0.36% of sulfur, and 20.53% of oxygen. The proximate analysis for this coal showed 6.56%, 48.23 %, and 45.21% of ash, volatile matter and fixed carbon, respectively. Coal samples were exposed to air during storage, handling, and preparation, which may have also influenced their biodegradability and bioavailability.

The nutrient amendments included a balance nutrient solution of tryptic soy broth – TSB (15 g/L), and lactate medium – LAC (1 g/L yeast extract, 6.67 mL/L sodium lactate, 1.23 g/L sodium acetate, 0.5 g/L ammonium chloride, 1 g/L potassium phosphate, 0.2 g/L magnesium sulfate, 0.1 g/L calcium chloride, and 0.5 g/L sodium sulfate). These nutrient media provided easily degradable carbon sources and appropriate nutrient compounds (e.g., nitrogen) to stimulate microbial growth and methane production (Opara, 2012). No pH buffer was used, and all of the nutrient media were close to a neutral pH.

5.3.3. Reactivation of Microbial Consortia

Promising microbial consortia were chosen after a three-phase screening program developed in Fuertez et al. (2017). Briefly, three phases of screening were needed to enrich microbial communities collected from various hydrocarbon-rich environments and locations characterized by natural methanogenesis, and to adapt those enriched microbes

to selected coal sources. In addition, the most favorable nutrient amendments for producing large amounts of methane under initial atmospheric exposure were identified. After twenty-four weeks of incubation, however, methane production dropped significantly in most of those mentioned samples.

Subsequently, an additional reactivation or recovery step was considered in this work to supply the nutrients required for growth and functionality of selected microbial samples. These samples were chosen on the basis of their methane production in the last stage of the described experimental program. The type and amount of nutrient solutions (i.e., 20 mL of TSB or LAC), coal material (i.e., 5 g of DCWC or MBT), and amount of inoculum (5 mL) were equal to those used to culture the consortia during their development (Fuertez et al., 2017). Gas production was periodically monitored. Methane and carbon dioxide concentration of each bioreactor headspace were measured every two and seven days over a nine-week period. A total of 13 measurements were carried out with gas chromatography.

5.3.4. Separation of Adapted Microbes

Following sixty-three day cultivation of the aforementioned samples, the entire content was well mixed and allowed to settle (Zhang et al., 2015). An initial aliquot (2.5 mL) of liquid phase was withdrawn. Additionally, a portion of coal residue and remaining liquid (5 mL) was retrieved and diluted. A volume of 2.5 mL of each sample (i.e., initial aliquot and dilute sample) were then mixed, and 5 mL were added into a bioreactor containing only 20 mL of fresh nutrient medium of the same type (i.e., TSB or LAC) used to culture the microbes during their development (Fuertez et al., 2017).

The previous procedure was carried out to obtain a representative sample of the microbial consortia from the enriched culture. Methane and carbon dioxide production were measured every two and seven days during thirty-five days of incubation to confirm the presence of active methanogenic populations after separation from the enriched cultures, and to broadly estimate methane production from added nutrient amendments. A total of 9 measurements were conducted using gas chromatography.

5.3.5. Converting Coal to Methane

After the thirty-five days of incubation in growth media, promising microbial consortia were used to evaluate their potential application to convert coal sources into methane as shown during their enrichment and adaptation stage (Fuertez et al., 2017). Coal biogasification under reduced nutrient amendment and salt presence was examined. Coal samples (bituminous Deer Creek Mine Waste coal – DCWC, subbituminous Miller Black Thunder coal – MBT) and fresh nutrient solutions (tryptic soy broth – TSB, lactate – LAC) corresponded to the same type used to culture the select consortium in Fuertez et al. (2017). Factor levels (i.e., percent of salt solution, nutrient amount, and percent of inoculum) were determined based on literature (Furmann, 2011; Furmann et al., 2012; Green et al., 2008; Harris et al., 2008; Jones et al., 2010; Opara, 2012; Opara et al., 2012; Orem, 2010; Papendick et al., 2011).

Therefore, six grams aliquots of pulverized coal (< 0.42 mm particle size) were added into bioreactors. It is acknowledged that less particle size of coal has an important effect on the extent of biological methane production (Green et al., 2008; Gupta and Gupta, 2014). Thus, the results presented here may represent a desired scenario. All samples were

exposed to air during storage, handling, and preparation. This may have influenced their bioavailability and biodegradability. In fact, this exposure is an important consideration taking into account the desirability of culturing effective methanogens in an aerobic field scale production scenario.

A total liquid volume of 25 mL was used. A total of 22.4% v/v of this volume corresponded to the stock nutrient solution (5.6 mL) with neutral pH, while a 10% corresponded to the microbial consortium (2.5 mL), which was previously centrifuged and washed with normal saline solution - 8.5 mg/cm^3 NaCl - to reduce remaining nutrients. An aliquot (1 mL) of this inoculum was used for plate counting on trypticase soy agar (TSA) plates prior to inoculation; aerobic, aerotolerant and culturable organisms were especially counted (Benson, 2002; Opara, 2012; Steubing, 1993). The balance of the total liquid volume (16.9 mL) in bioreactors corresponded to sterile salt solution (NaCl) whose final concentration was computed as 6.6 mg/cm^3.

Gas chromatography was used to determine methane and carbon dioxide concentrations. Measurements were conducted 10 times during one hundred and twenty-two days of incubation at 23°C. This period of incubation was considered to allow mesophilic methanogens present in the cultures to grow and produce significant methane gas (Del Real, 2007; Ferry, 1993; Zhang et al., 2015; Zupančič and Grilc, 2012). The above description of bioreactors' composition corresponded to the microbial samples labeled as "S1" in Table 12.

Two control samples were included for comparative purposes. The first control sample (i.e., C1 in Table 12) corresponded to coal (6 g) plus nutrient amendment (5.6 mL) and salt solution (19.4 mL) to assess gas production from native microbes and/or absorbed

gas present in coal samples. The second control sample (i.e., C2 in Table 12) included salt solution (22.5 mL) plus microbial consortia (2.5 mL) to assess gas from degradation of the dead cells by the remaining populations and/or possible presence of nutrients after washing. The final salt concentration in these controls was also 6.6 mg/cm^3 NaCl.

Moreover, for practical purposes the overall theoretical conversion of nutrient organic content to methane and carbon dioxide was estimated (Equation 2). This allowed to evaluate the gas production that could have come directly from nutrient utilization (Buswell and Neave, 1930; Kayhanian et al., 2007; Opara et al., 2012; Opara, 2012).

$$C_cH_hO_oN_nS_s + yH_2O \rightarrow \mathrm{x}CH_4 + (c-x)CO_2 + nNH_3 + sH_2S \qquad (2)$$

where x = 0.125(4c+h-2o-3n-2s), and y = 0.25(4c-h+2o+3n+2s)

5.3.6. Exploring Community Level Metabolic Profiling

Considering that each microbial species and microbial population has a specific and usually unique set of carbon compounds they can utilize as substrate, BIOLOG ECO™ plate system was used as a screening tool to broadly explore the pattern of carbon utilization (community level physiological profiling - CLPP) of promising microbial consortia (Opara, 2012). CLPP represents a sensitive and rapid method for assessing the potential metabolic diversity of microbial communities (Garland, 1997; Preston-Mafham et al., 2002).

An ECO plate consists of 96 wells, each well contains one of 31 carbon sources, which are present in triplicates for reproducibility purposes. The remaining three wells are

control wells. Formation of purple color occurs when the microbes can utilize the carbon source and begin to respire. The respiration of the cells in the community reduces a tetrazolium dye that is included with the carbon source. This colorimetric reaction can be monitored with time, indicating which of the carbon sources can be utilized by the consortium (Opara, 2012; Preston-Mafham et al., 2002).

It is noted that after the separation of adapted microbial consortia from enriched cultures (i.e., coal sources plus nutrient amendments), they were maintained in fresh nutrient media of the same type used during their development (Fuertez et al., 2017). 5 mL of inoculum were periodically transferred (every thirty-five days) into bioreactors containing only 20 mL of fresh nutrient medium (i.e., TSB, LAC or ACE). These microbial samples were set aside in the dark without agitation at 23°C. This procedure was considered to keep microbial populations active for further analysis.

After two transferences of microbial consortia in nutrient media, samples were used for experimentation. Dilution series were performed until the concentration of approximately 10^4 cfu/mL for each consortium. Plate counting on trypticase soy agar (TSA) plates was used to determine the concentration of microbes. Aliquots of 100 µL of consortia were transferred into each well of the BIOLOG ECO™ plate (Opara, 2012; Zak et al., 1994). Plates were incubated at 23°C. Color development was periodically monitored every twenty-four hours for seven days (Garland, 1997). This experiment was carried out once during this study.

5.3.7. Microbial Community Present in Culture

After six transferences of microbial consortia (i.e., adapted microbes) in fresh nutrient media, aliquots (20 mL) of two microbial samples incubated at 23°C were sent to the Environmental Engineering and Microbiology Laboratory at the University of Utah for DNA extraction. DNA was extracted and purified using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) as instructed in the manufacturer's protocol. Subsequently, analysis and identification of microbial communities through 16S rRNA gene-based amplicon sequencing were performed at the Research and Testing Laboratory (Lubbock, TX, USA). A matching of 97% was reported for the identification of microbial populations (Wu et al., 2016).

5.3.8. Gas Chromatography

The headspace methane and carbon dioxide concentrations in each bioreactor were determined with a Hewlett Packard HP6890 GC system (Palo Alto, CA) with a GS-GasPro PLOT column containing a bonded silica-based stationary phase. A flame ionization detector (FID) and thermal conductivity detector (TCD) were connected in series to analyze organic compounds and inorganic gases. Helium was used as the gas carrier. The temperature program used for this study began with 35°C for 4 min to allow for carbon dioxide and methane elution and was then increased by 25°C min^{-1} to 260°C. Scotty Analyzed Gases were used as standards to build calibration curves for methane and carbon dioxide. GC ChemStation (Agilent Technologies) computer software was used.

5.4. Results and Discussion

The potential application of microbial consortia for biogasification of coal under initial atmospheric exposure was evaluated. Selected microbial consortia were incubated with reduced concentration of nutrient amendments and salt presence as a possible scenario and foresee the elevated costs of nutrient addition in large-scale operations. Methane was mainly monitored over time as described in the experimental program of this study. Gas concentrations were reported in parts per million (ppm), and approximate values of sft^3/ton of coal were determined (Opara, 2012; Opara et al., 2012). Units of sft^3/ton are meaningful units for assessing commercial viability of coalbed methane gas production (Park and Liang, 2016).

Results for the microbial samples that produced the highest methane and carbon dioxide concentrations are included in this work. For practical purposes, these samples have been labeled and abbreviated according to the sequence of the screening-protocol shown in Fuertez et al. (2017). BIOLOG ECO™ plates were used as tool to broadly explore the metabolic profiling of developed microbial consortia. Moreover, microbial communities of two promising consortia were identified, allowing to consider their likely role in coal biogasification.

5.4.1. Reactivation of Microbial Consortia

After enrichment and adaptation of microbial consortia (i.e., microbial communities retrieved from coal and lake sediments) to selected coal sources, large methane concentrations were obtained after two weeks of incubation (e.g., 873,000 ppm, 154 sft^3/ton). However, those initial samples were kept under observation up to twenty-

four weeks. After this period, methane concentrations decreased significantly in most of the evaluated bioreactors (e.g., less than 4500 ppm, 0.79 sft^3/ton) (Fuertez et al., 2017).

As part of this study, promising microbial consortia were chosen. These microbial samples were selected on the basis of their methane production at two weeks of incubation in mentioned experimental program. Subsequently, these samples were reactivated. Once the microbial communities were inoculated in the bioreactors containing fresh nutrient amendments and coal material as described in Materials and Methods section of Chapter 5, longer time was needed to obtain large methane concentrations in comparison to preliminary results obtained during microbial consortia development (Figure 8).

In average, between twenty-one and thirty-four days of incubation were needed to reach the largest methane concentrations (Table 13). It is noted that headspace gas concentrations were measured every two and seven days over a nine-week period. Figure 9 depicts carbon dioxide production associated with the maximum methane concentration measured for the selected samples after reactivation.

Generated headspace gas concentrations reached up to 656,900 ppm (116 sft^3/ton) for methane (Figure 8), and 51,880 ppm (9.1 sft^3/ton) for carbon dioxide (Figure 9) with the sample ETL LAC LAC DCWC, for instance. This microbial consortium was obtained through a sequential enrichment of microbial populations from East Texas Lignite coal – ETL with lactate solution – LAC and a final adaptation stage to bituminous Deer Creek Mine Waste coal – DCWC (Fuertez et al., 2017). It is likely that the low initial cell concentration of the inoculum for reactivation, or changes of microbial communities may explain why cultures run at the same conditions at different times yielded different gas production in different incubation periods (Green et al., 2008). After reactivation, however,

viable methanogens were present in the enriched samples as shown by the methane production as a sign of methanogenic activity (Green et al., 2008; Rathi et al., 2015).

5.4.2. Separation of Adapted Microbial Consortia

After the separation of adapted microbes (microbial consortia) from enriched cultures (i.e., consortia plus coal material and nutrient amendments), these were fed with 100% v/v of fresh nutrient amendments (i.e., TSB or LAC) and were monitored for methane and carbon dioxide production every two and seven days up to thirty-five days of incubation. It is noted that methane concentration dropped after thirty-three days in most of the evaluated samples. This suggested a possible accumulation of intermediary or toxic compounds, microbial methane oxidation (Fuchs et al., 2016; Megonigal et al., 2013), and/or that limited amounts of electron donors (i.e., acetate or H_2) were not continuously produced to support methanogenesis. Gas production, however, showed that methanogenic populations were satisfactorily retrieved from enriched cultures and were kept active (Figure 10).

In summary, the maximum methane concentrations measured with nutrient medium (e.g., 68,200 ppm for consortium JR2 TSB TSB DCWC) were less than those gas concentrations obtained during microbial consortia development and subsequent reactivation with coal material. Microbial consortia produced more methane in cultures when coal was present versus cultures when it was absent. This behavior has been also reported by other researchers (Papendick et al., 2011; Ritter et al., 2015; Zhang et al., 2016).

Table 14 indicates the time when maximum methane concentrations were

measured. Considering these concentrations, low production of methane might be obtained as direct result of nutrient amendments conversion during coal biogasification. However, appropriate control samples (e.g., microbial consortia plus nutrient amendments) should be used for this purpose. It has been considered that nutrient addition is an important element to stimulate microbial growth, supplying essential compounds (e.g., phosphorous, nitrogen) that may not be directly available in the coal sources (Bao et al., 2016; Opara, 2012; Opara et al., 2012; Ritter et al., 2015).

5.4.3. Converting Coal to Methane

After incubation in growth media, the promising microbial consortia: JR2 TSB TSB DCWC (JTTD), MBT TSB TSB DCWC (MTTD), MBT LAC TSB MBT (MLTM), UL4 TSB TSB MBT (UTTM), and ETL LAC LAC MBT (ELLM) were selected to assess their capabilities of converting coal sources (i.e., bituminous Deer Creek Mine Waste coal – DCWC and subbituminous Miller Black Thunder coal – MBT samples) into methane under low concentration of nutrient amendments (e.g., 22.4% v/v or 3.36 mg/cm^3 TSB), and NaCl concentration of 6.6 mg/cm^3. Methane and carbon dioxide concentrations were monitored up to one hundred and twenty-two days of incubation. Carbon dioxide concentrations showed a decreasing trend, especially in the bioreactors formed by salt solution, nutrient amendment, coal source and microbial consortia (i.e., sample "S1" in Table 12), and control samples formed by nutrient amendments, salt solution and coal sources (i.e., sample "C1" in Table 12). Concentrations of carbon dioxide for the above samples varied between 7,000 ppm (1.0 sft^3/ton) and 100,000 ppm (14.6 sft^3/ton) during experimentation. This behavior might be attributed to the complexity of carbon dioxide chemistry (Ibanez et al.,

2007).

In comparison to the bioreactors labeled as "S1" in Table 12 (i.e., gas production curves depicted in blue and green colors in Figure 11), less carbon dioxide (< 7,800 ppm) and methane (< 2,300 ppm) concentrations were obtained in control samples labeled as "C2" in Table 12 (i.e., gas production curves depicted in purple and orange in Figure 11). Gases generated from these controls might have come from degradation of dead cells by the remaining populations, and/or remaining nutrients after washing procedure (Opara et al., 2012).

During bioconversion of coal in the bioreactors labeled as "S1" (Figure 11) and related to the microbial samples JTTD, MTTD, MLTM, UTTM, and ELLM, less methane production (< 200,000 ppm) was obtained in comparison to the values measured after reactivation (Figure 8). These results may infer the influence of various factors, such as microbial density (Table 15), salinity, changes in microbial populations over time, and/or concentration of nutrient amendments (Park and Liang, 2016; Ritter et al., 2015; Zhang et al., 2015). For instance, large concentrations of nutrients can accelerate and increase gas production from coal stimulating microbial populations to higher proportion (Opara et al., 2012; Zhang et al., 2015, 2016). Thus, a large gas production might be obtained with high concentrations of the microbial consortia's optimal nutrient amendments and an appropriate environment. In fact, this behavior was observed when our microbial consortia were enriched and adapted to select coal samples during their development (Fuertez et al., 2017), and subsequent reactivation stage (Figure 8) using 100% v/v of nutrient amendments (e.g., TSB, LAC). However, it is noted that nutrients solutions should be added in such an amount that they are kept inexpensive as possible while maintaining their

stimulating effect to be considered at commercial scales (Bao et al., 2016; Zhang et al., 2016).

Opara et al. (2012) reported methane production up to 0.06 sft^3/ton and 21 sft^3/ton with addition of 10% (7.7% v/v of total liquid) and 50% (38.5% v/v of total liquid) nutrient amendments, respectively, using aerotolerant microbial consortia and bituminous Deer Creek Mine Waste coal (< 75 µm particle size) at 23°C and thirty days of incubation period. A similar behavior was also observed when lignite and coal waste materials were used by those researchers (Zhang et al., 2016).

Additionally, it is considered that the salt concentration used in the bioreactors (6.6 mg/cm^3 NaCl) could have had a significant effect on microbial consortia, inhibiting microbial growth, and reducing gas production to some extension (Liu and Boone, 1991; Schnürer and Jarvis, 2010). A detrimental effect on methane production was confirmed in subsequent studies. Bioaugmentation strategy, however, was proven to be effective in enhancing methane production from coal material using the microbial consortia (i.e., gas production curves depicted in blue and green colors in Figure 11). A lag phase between twenty and forty days was initially present during this process. It is likely that this period corresponded to the transition between the use of added nutrients and coal utilization, and/or an adaptation stage to the salt concentration present in the medium (Green et al., 2008).

A high rate of methane production was subsequently observed in all evaluated cultures consortia (i.e., gas production curves depicted in blue and green colors in Figure 11). Interestingly, the large methane concentrations were not maintained during the whole period of incubation. After the maximum methane concentrations were reached (Figure

11), a decreasing trend was followed for some of the evaluated microbial samples. This scenario of methane content increasing and subsequently decreasing with time has been reported under strict anaerobic conditions (Papendick et al., 2011; Zhang et al., 2015, 2016). As a possible explanation, microbial methane oxidation could have occurred (Megonigal et al., 2013). It is also likely that available nutrients or trace elements may have depleted over time. This depletion could have led to a decrease in the microbial populations.

Other reasons include the accumulation of toxic or inhibitory degradation products. Finally, it is speculated that only limited amounts of electron donors (i.e., acetate or H_2) were available or were not continuously produced to support methanogenesis. Further investigation is still needed to give an exact explanation to the above behavior, and to propose effective alternatives that ensure a continuous and enhanced methane production.

The maximum values of methane production, carbon dioxide, and initial cell concentration of the microbial consortia inoculated into bioreactors ("S1" samples in Table 12) with nutrient amendments, salt solution and coal material (i.e., gas production curves depicted in blue and green colors in Figure 11) are presented in Table 15. The theoretical maximum gas generation values from nutrient amendments at 22.4% v/v (e.g., 3.36 mg/cm^3 TSB) were estimated between 820 ppm and 2,350 ppm for methane, and from 660 ppm to 1,570 ppm for carbon dioxide production (Buswell and Neave, 1930; Kayhanian et al., 2007; Opara 2012; Opara et al., 2012). In comparison to the maximum gas production experimentally obtained (Table 15), a small fraction (1-9%) of the measured gases might be attributed to the direct nutrient conversion.

5.4.4. Exploring the Community Level Metabolic Profiling

Table 16 summarizes the results of metabolic profiling of promising microbial after seven days of incubation. Consortium ETL LAC LAC MBT (ELLM), was the most versatile since it used 28 carbon sources. Consortia RH TSB TSB MBT (RTTM), and MBT TSB TSB DCWC (MTTD) were also diverse, using 25 and 22 carbon sources, respectively. Consortium MBT LAC TSB MBT (MLTM) used only 18 carbon sources, while consortia JR2 TSB TSB DCWC (JTTD) and ETL LAC LAC DCWC (ELLD) both used 15 carbon sources. Finally, consortium UL4 TSB TSB MBT (UTTM) was the least diverse. It only used 6 carbon sources.

Except the UL4 TSB TSB MBT (UTTM) microbial sample, microbial consortia metabolized almost all of the carbohydrates, amino acids, and phosphate carbon sources. The most diverse microbial communities (e.g., ELLM, RTTM) also used carboxylic acids, and polymers as more complex carbon structures included in the BIOLOG ECO^{TM} plates system. These results broadly show the ability of the microbial consortia to metabolize different carbon compounds.

Previous observations can be quantified using the community metabolic diversity (CMD) factor (Figure 12). This factor represents the total number, but not the type of substrates that have been effectively metabolized by a given microbial community (Opara, 2012; Zak et al., 1994). As shown in Figure 12, microbes from ETL LAC LAC MBT (ELLM) sample rapidly metabolized most of the carbon sources. In just forty-eight hours, 23 (74.2%) of the 31 compounds were used. Microbial consortia RH TSB TSB MBT (RTTM), on the other hand, spent ninety-six hours to metabolize 24 (77.4%) carbon sources. Different rates of carbon utilization were manifested, which may be associated to

the number and type of microbial species present in each consortium (Preston-Mafham et al., 2002).

In addition to the above observations, a percent similarity can be found for the evaluated consortia (Figure 13). This percentage indicates how functionally similar given two consortia are, and it is computed considering the number substrates that have been simultaneously metabolized by both consortia (Opara, 2012). All possible combinations among microbial communities from the selected samples are shown in Figure 13. As depicted, the microbial consortia RH TSB TSB MBT (RTTM) and ETL LAC LAC MBT (ELLM) were nearly 77.4% similar at the end of the incubation period. It is probably that these consortia retrieved from coal sources after being enriched and adapted, comprised similar microbial populations that are able to use subbituminous Miller Black Thunder coal – MBT as substrate.

On the other hand, a percent similarity less than 68% was observed for the other combinations between microbial consortia. In overall, there was less percent similarity between microbial consortia cultured from noncoal environments (i.e., JTTD, UTTM) and those microbes obtained directly from coal environments. The lowest percentages were computed for combinations with UTTM sample. This microbial consortium was obtained after the enrichment of microbial communities from Utah Lake – UT with TSB and an adaptation stage to Miller Black Thunder coal – MBT (Fuertez et al., 2017).

It is believed that microbial populations present in UTTM were different from those microbes in the other consortia, showing the lowest functional similarity. In fact, the microbial populations in UTTM were retrieved from an aquatic environment characterized by natural methanogenesis with nonnative coal species. Additionally, it is also likely that

microorganisms could have changed as result of differential growth and competition (Green et al., 2008; Preston-Mafham et al., 2002), and/or were inhibited for any toxic compound into the wells (Preston-Mafham et al., 2002). Similar trends were observed when Opara (2012) evaluated five aerotolerant microbial consortia. Those consortia were obtained by combining the best carbon dioxide and methane producers from various sources (i.e., bituminous coal and waste coal, lake sediments, wetland sediments, river sediments, digester sludge, oil seep and gas well samples). The microbial populations obtained here and Opara's microbial consortia showed biodegradation of complex carbon structures.

As described in Materials and Methods of Chapter 5, two transferences of microbial consortia in fresh nutrient media were used prior this test. Thus, a broad view of the metabolic diversity was obtained for the consortia evaluated. It is noted that previous experiments (i.e., separation of microbial consortia and coal conversion to methane) were sequentially carried out. After a long period (approximately two months) of incubation and transference of microbes into fresh nutrient media, the metabolic profiling was obtained.

A simultaneous evaluation of coal bioconversion with nutrient amendments, determination of metabolic profiling, and identification of microbial communities present in the bioreactors would revel improved insights of the biogasification process with the selected microbial consortia. Proposed experimental program would enable to determine the relation between gas production from selected coal types, nutrient amendments, microbial populations, and their metabolic profiling.

5.4.5. Microbial Community Present in Methanogenic Consortia

Biogasification of coal requires an interactive participation of various microorganisms. Among them, three major metabolic groups are recognized: hydrolytic and fermentative bacteria, acetogenic bacteria, and methanogenic archaea. Initially, complex organic compounds in coal are decomposed to simpler molecules (e.g., acetate, long fatty acids, carbon dioxide, hydrogen, methane) by fermentative microorganisms. Fatty acids, alcohols, some aromatic, and amino acids are converted to hydrogen, carbon dioxide, and acetate by H_2-producing acetogens while H_2-using acetogenic bacteria consume H_2 and CO_2 to produce more acetate. Finally, simple molecules are converted to CH_4 by methanogens that belong to the domain of archaea (Meslé et al., 2013; Zhang et al., 2015).

The diversity assay revealed that the enriched consortium JR2 TSB TSB DCWC (JTTD) contained 99.92% of bacterial strains, 0.056% of archaea, and 0.028% of others (Figure 14). This consortium was obtained after culturing native microbial populations from Jordan River – UT (a noncoal environment) with TSB and a final adaptation stage to bituminous Deer Creek Mine Waste coal (Fuertez et al., 2017).

The enriched consortium RH TSB TSB MBT (RTTM) contained 98.26% of bacteria and 1.74% of archaea (Figure 14). Microbial populations of this consortium were obtained by enrichment of lignite Red-Hills coal – RH with TSB and an adaptation stage to subbituminous Miller Black Thunder coal – MBT (Fuertez et al., 2017).

Similar percentages of total population for enriched and adapted consortia on coal can be found in literature (Meslé et al., 2013; Zhang et al., 2015). It is noted that microbial consortia (i.e., adapted microbes) used for experimentation were maintained in nutrient

solutions (Lavania et al., 2014; Rathi et al., 2015). After six transferences of microbes into fresh nutrient media, aliquots of the maintenance culture were used for DNA extraction and subsequent identification. Thus, possible changes of microbial communities could have been present (Green et al., 2008). However, a broad view of the evaluated microbial consortia composition was obtained.

Among all of the bacteria identified in the microbial consortium JR2 TSB TSB DCWC (JTTD), the major populations were (Figure 15): *Bacteroides sp., Petrimonas sp., Clostridium beijerinckii, Clostridium sp., Clostridium propionicum, Lachnoclostridium* spp., *Acetonema spp., Dehalobacter sp., Anaerofilum sp., Syntrophomonas sp., Aquamicrobium sp., Advenella spp., Comamonas sp., Thauera sp., Desulfovibrio sp., Citrobacter sp., Pseudomonas stutzeri,* and *Aminobacterium sp.* Among all identified archaea, the dominant populations belonged to the genus *Methanofollis*.

With regard to the consortium RH TSB TSB MBT (RTTM), the most abundant bacteria populations were (Figure 16): *Cellulomonas sp., Paenibacillus motobuensis, Clostridium beijerinckii, Clostridium sp., Lachnoclostridium spp., Desulfitobacterium hafniense, Sporomusa spp., Tissierella praeacuta, Tissierella sp., Achromobacter sp., Citrobacter sp.,* and *Stenotrophomonas sp.* In terms of archaea identification, this consortium comprised only one species: *Methanobacterium sp.*

Above results showed that adapted microbial populations contained methanogens form the order Methanomicrobiales and Methanobacteriales. Additionally, different bacterial phyla, among which Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria dominate, have been found either in coal and oil settings (Colosimo et al., 2016; Guo et al., 2014; Heat et al., 2014; Meslé et al., 2013). The bacterial and archaea genera identified in

the microbial consortia, their metabolic potential and possible role in the degradation of the complex organic matter is described in Table 17. It is noted that there exists little or limited metabolic data on their actual ability to degrade coal (Meslé et al., 2013). As shown in the Table 17, some aerobes and microorganisms that exhibit certain tolerance to oxygen were present. While is unclear why some of these microbes, which require oxygen to degrade organic matter (e.g., cellulose), have been found in anaerobic environments, their role is still considered relevant to metabolize highly complex, degradation recalcitrant, organics as the sole carbon and energy source (Colosimo et al., 2016; Head et al., 2014; Meslé et al., 2013).

Interestingly, obligate anaerobes were also identified in the evaluated microbial consortia. Our results infer the tolerance of microbial communities to periodic oxygen exposure during their enrichment and adaptation stage to selected coal materials (Fuertez et al., 2017), and the subsequent protocols carried out in this study. During experimentation, cultures were exposed to initial oxygen environments and still exhibited methanogenic activity. Microbial communities identified in the developed microbial consortia showed the presence of metabolic groups (Table 17) commonly observed in the bioconversion of coal (Colosimo et al., 2016; Meslé et al., 2013).

Complementing Opara's work (Opara, 2012; Opara et al., 2012), a complex experimental matrix was used to select the best combination of nutrient amendments, coal sources, and microbial populations formed by aerobic, facultative and strict anaerobes able to co-exist and metabolize select coal material into methane (Fuertez et al., 2017). The microbial populations of promising microbial consortia were subsequently identified in this study. This allowed to verify the presence of important elements/players for the coal

biogasification under periodic oxygen exposure. It is unknown whether Opara's aerotolerant consortia contained novel or known strains of aerotolerant methanogens (e.g., *Methanobacterium thermoautotrophicum, Methanobacterium bryantii*) (Kato et al., 1997; Kiener and Leisinger, 1983) since those consortia were partially characterized. Finally, due to the short time of incubation used by Opara et al. (2012) (i.e., thirty days), it is not possible to determine whether the reported maximum methane production was obtained, and whether this gas production could be kept over time. This behavior was explored in this subsequent work.

5.4.6. Oxygen Tolerance

Within a methane generating process, the importance of oxygen concentration varies considerably when different microbial communities are present. Some microbes are very sensitive to oxygen exposure. Others can survive quite low oxygen concentrations, while others grow better whether oxygen is in the surroundings (Schnürer and Jarvis, 2010). The coexistence of anaerobic and aerobic microorganisms in anaerobic environments, such as coal deposits and oil reservoirs has been observed, attributing generally the presence of aerobes to the possible entrance from surface environments (Head et al., 2014; Meslé et al., 2013).

Methanogenesis is considered to be drastically inhibited by oxygen. Methanogens are commonly known as strict (obligate) anaerobes that metabolize only in anaerobic environments and are extremely sensitive to this gas (Wolfe, 2011). Thus, strict anaerobic techniques are usually suggested for their study in laboratory settings (Furmann, 2011; Furmann et al., 2013; Green et al., 2008; Wolfe, 2011). However, some methanogens are

not necessarily as oxygen sensitive as has been thought. It is plausible that some methanogenic populations have evolved to handle different levels of oxygen because their natural habitats are exposed to various levels of this gas throughout long periods of time (Botheju and Bakke, 2011; Jarrell, 1985; Kato et al., 1997; Kiener and Leisinger, 1983; Kirby et al., 1981; Megonigal et al., 2013). Reported methanogens that have exhibited certain tolerance to oxygen exposure mainly belong to the orders Methanomicrobiales and Methanobacteriales (Jarrell, 1985; Kato et al., 1997; Kiener and Leisinger, 1983; Kirby et al., 1981). Methanogens identified in our study (i.e., *Methanobacterium sp*., *Methanofollis spp*.) were also related to those orders, raising the number of known methanogenic populations able to tolerate periodic atmospheric exposure.

It is believed that aerobic and facultative microorganisms present in our consortia (e.g., *Cellulomonas, Aquamicrobium, Achromobacter, Advenella, Comamonas*, *Thauera*, *Pseudomonas, Paenibacillus, Citrobacter*) could rapidly consume oxygen, creating favorable conditions for the development of obligate anaerobes (e.g., *Bacteroides, Petrimonas, Clostridium*, *Tyzzerella, Lachnoclostridium, Anaerofilum, Syntrophomonas, Acetonema, Dehalobacter, Desulfitobacterium, Tissierella, Desulfovibrio*, *Aminobacterium*). Thus, a temporary air leakage to the methane generating process can be handled since the aerobic and facultative microorganisms are able to reduce the incoming oxygen to a low level (e.g., 2 µg/L). This feature and some intrinsic oxygen tolerance of anaerobes organisms present in the cultures could have allowed the coal biogasification to proceed (Ali-Shah et al., 2014; Ibanez et al., 2007; Kato et al., 1997; Schnürer and Jarvis, 2010).

5.5. Conclusions and Implications

The results of this study infer that methanogenic microbial consortia can be used for coal biogasification. These consortia should tolerate oxygen exposure during culturing, storage, and injection into a target coalbed (in situ application). For ex situ applications, these microbial consortia could be high graded for use on coal waste heaps or bioreactors where oxygen exposure is anticipated. The microbial conversion of coal into methane, if it could be successfully implemented at large scale, would be an optimal use of coal as an abundant natural source (Zhang et al., 2016).

Furthermore, it was demonstrated that these consortia can be successfully reactivated and/or recovered after a long time of incubation, allowing to restimulate microbial populations and subsequently to continue the gas production from selected coal sources. In addition, as shown by Bum-Han et al. (2017), the developed microbial consortia can be encapsulated and revitalized for their application in in situ operations. This highlights the capability of microbial consortia to be implemented in large-scale operations.

The fact that these microbial consortia can still generate significant amount of gas under low concentration of nutrient amendments, makes of these consortia an attractive low-cost biological complement for coal biogasification.

The feasible coexistence of aerobes, facultative, and strict anaerobes in these consortia increases their potential to be implemented at large scale operations. This potential is also reflected through their pattern of carbon utilization, showing a significant ability to metabolize complex carbon structures. This possibility adds to the arsenal of biodegradable capacities and potentially opens up new applications in environmental

technology.

The observed decreasing trends in methane content over time demands a deeper investigation. As other researchers have suggested (Zhang et al., 2015, 2016), it is still required to characterize each bioreactor in order to obtain a better explanation of the described behavior. Thus, effective alternatives or strategies can be proposed to maintain a stable and continuous methane production at laboratory scale initially.

Table 12. Bioreactors configuration for coal conversion test

Item	S1	C1	C2
Microbial consortium	✓		✓
Salt solution	✓	✓	✓
Nutrient amendment	✓	✓	
Coal source	✓	✓	

Table 13. Time of maximum methane production after reactivation

Sample	JTTD	RTTM	ELLD	MLTM	UTTM	ELLM	MTTD
Time (days)	34	21	21	19	21	34	34

Table 14. Time of maximum methane production from added nutrients

Sample	JTTD	RTTM	ELLD	MLTM	UTTM	ELLM	MTTD
Time (days)	33	33	33	25	33	12	12

Table 15. Maximum methane, related carbon dioxide production, and initial cell concentration

Sample		JTTD	MTTD	UTTM	MLTM	ELLM
Time	days	61	70	70	70	38
Maximum CH_4	ppm	95,700	26,350	37,340	66,980	3,750
	sft^3/ton	14	3.9	5.5	9.8	0.6
CO_2	ppm	37,560	53,400	66,320	97,530	40,450
	sft^3/ton	5.5	7.8	11.6	14.3	5.9
Initial cell concentration	cfu/mL	7.2×10^6	1.8×10^6	5.8×10^7	1.5×10^6	1.1×10^6

Table 16. Summarized results of metabolic profiling

Compound classes	**Microbial consortia**						
	JTTD	RTTM	ELLD	UTTM	ELLM	MTTD	MLTM
Polymers							
Tween 40		✓	✓	✓	✓	✓	
Tween 80		✓	✓	✓	✓	✓	✓
α-cyclodextrin					✓		
Glycogen					✓	✓	
Carboxylic acids							
D-galactonic acid-gamma-lactone		✓	✓		✓	✓	✓
D-galacturonic acid	✓	✓	✓		✓	✓	✓
2-Hydroxy benzoic acid							
4-Hydroxy benzoic acid		✓		✓	✓		
γ-hydroxy butyric acid					✓		
D-glucosaminic acid		✓			✓		
Itaconic acid		✓				✓	
α-keto butyric acid							
D-malic acid	✓	✓			✓	✓	
Pyruvic acid methyl ester	✓	✓	✓	✓	✓	✓	✓
Carbohydrates							
β-methyl-D-glucoside	✓	✓	✓		✓	✓	✓
D-xylose	✓	✓			✓	✓	✓
i-erythritol		✓		✓	✓	✓	✓
D-mannitol	✓	✓	✓		✓	✓	✓
N-acetyl-D-glucosamine	✓	✓	✓		✓	✓	✓
D-cellobiose	✓	✓	✓		✓	✓	✓
α-D-lactose	✓	✓	✓		✓	✓	✓
Amino acids							
L-arginine		✓			✓		
L-asparagine	✓	✓	✓		✓	✓	✓
L-phenylalanine		✓		✓	✓	✓	✓
L-serine	✓	✓	✓		✓	✓	✓
L-threonine		✓			✓	✓	✓
Glycyl-L-glutamic acid	✓	✓	✓		✓	✓	✓
Amines							
Phenylethylamine					✓		
Putrescine	✓	✓			✓		
Phosphate carbon							
Glucose-1-phosphate	✓	✓	✓		✓	✓	✓
D,L-α-glycerol phosphate	✓	✓	✓		✓	✓	✓
Total sources used	*15*	*25*	*15*	*6*	*28*	*22*	*18*

Table 17. Bacterial and archaeal genera found in microbial consortia

	Genera	Main metabolism	Function	Reference
Bacteria				
Actinobacteria	*Cellulomonas*	Aerobic or facultative anaerobe. Cellulolytic, metabolism of poor water soluble organic compounds. Oxygen may be required to degrade cellulose.	Fermenters	Meslé et al. (2013); Stackebrandt and Schumann (2015)
Bacteroidetes	*Bacteroides* *Petrimonas*	Obligate anaerobes. Metabolism of organic acids and polymers.	Fermenters	Meslé et al. (2013)
Firmicutes	*Paenibacillus*	Facultative anaerobe. Hydrolytic digestion of carbohydrates. Acetate production or oxidation.	Fermenters, acetogens	Priest (2015)
	Clostridium *Tyzzerella* *Lachnoclostridium,* *Anaerofilum,* *Syntrophomonas* *Acetonema* *Sporomusa*	Mostly obligate anaerobes, but tolerance to oxygen varies. Hydrolytic digestion of macromolecular compounds. Production of organic acids, alcohols. Acetate production or oxidation.	Fermenters, acetogens	Drake and Gössner (2015); Massett et al. (2012); Meslé et al. (2013); Rainey et al. (2015); Rainey, (2015a, 2015b); Sekiguchi (2015); Yutin and Galperin (2013)
	Dehalobacter	Obligate anaerobes. Fermentative dehalogenation; use of chlorinated aliphatic and aromatic compounds. Competition with methanogens for hydrogen and nutrients. Acetate production or oxidation.	Fermenters	Holliger (2015)
	Desulfitobacterium	Obligate anaerobes, but some strains aerotolerant. Fermentative dehalogenation.	Fermenters	Lupa and Wiegel (2015); Villemur et al. (2006)
	Tissierella	Obligate anaerobes. Use of certain aminoacids and formate. Production of acetate, ammonia, hydrogen and carbon dioxide.	Fermenters	Nolla-Ardevol et al. (2015); Shah and Hookey (2015)

Table 17 continued

	Genera	Main metabolism	Function	Reference
Bacteria				
α-proteobacteria	*Aquamicrobium*	Aerobes. Degraders of water-insoluble compounds (polyaromatic hydrocarbons). Organic pollutants.	Fermenters	Meslé et al. (2013); Wu et al. (2014)
β-proteobacteria	*Achromobacter, Advenella Comamonas Thauera*	Aerobes. Possible anaerobe respiration with nitrate reduction. Saturated and aromatic hydrocarbon degraders. Use of organic acids and amino acids, hydrogen-utilizing. Few carbohydrates used.	Fermenters	Barra et al. (2009); Busse and Auling (2015); Heider, and Fuchs (2015); Meslé et al. (2013); Willems and Gillis (2015)
δ-proteobacteria	*Desulfovibrio*	Obligate anaerobes. Sulfate reducers, hydrocarbon degradation, and organic acids fermentation. Acetate production. Few carbohydrates used.	Fermenters	Kuever et al. (2015); Meslé et al. (2013)
γ-proteobacteria	*Citrobacter Pseudomonas Stenotrophomonas*	Facultative anaerobes or aerobes. In some cases, nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. Hydrocarbon degraders.	Fermenters	Frederiksen, (2015); Head et al. (2014); Meslé et al. (2013); Palleroni, (2015a, 2015b)
Synergistetes	*Aminobacterium*	Obligate anaerobes. Fermentation of a limited range of amino acids.	Fermenters	Baena et al. (2015)
Archaea				
Methanobacteriales	*Methanobacterium*	Hydrogenotrophic. Obligate anaerobe. Some strains can use formate, secondary alcohols, or CO.	Methanogen	Boone (2015); Meslé et al. (2013)
Methanomicrobiales	*Methanofollis*	Hydrogenotrophic. Obligate anaerobe. Substrates for growth and methane production are H_2/CO_2 or formate; possible use of 2-propanol/CO_2, 2-butanol/CO_2, and cyclopentanol/CO_2	Methanogen	Meslé et al. (2013); Zellner and Boone (2015)

This table was adapted from Meslé et al. (2013).

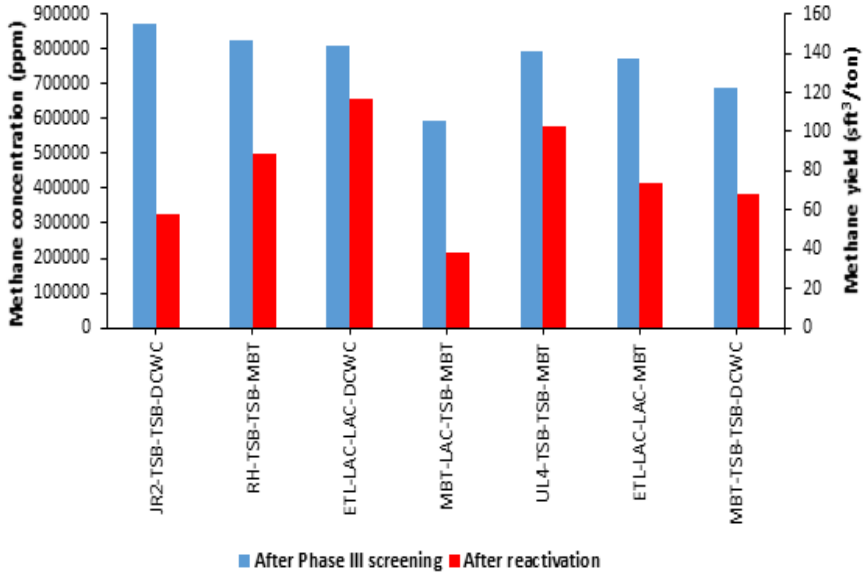


Figure 8. Comparation of methane concentration after reactivation

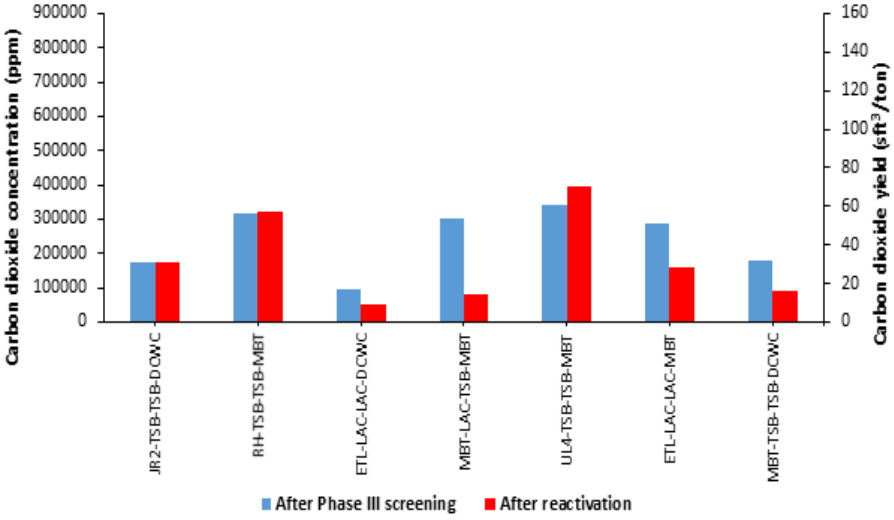


Figure 9. Comparation of carbon dioxide concentration after reactivation

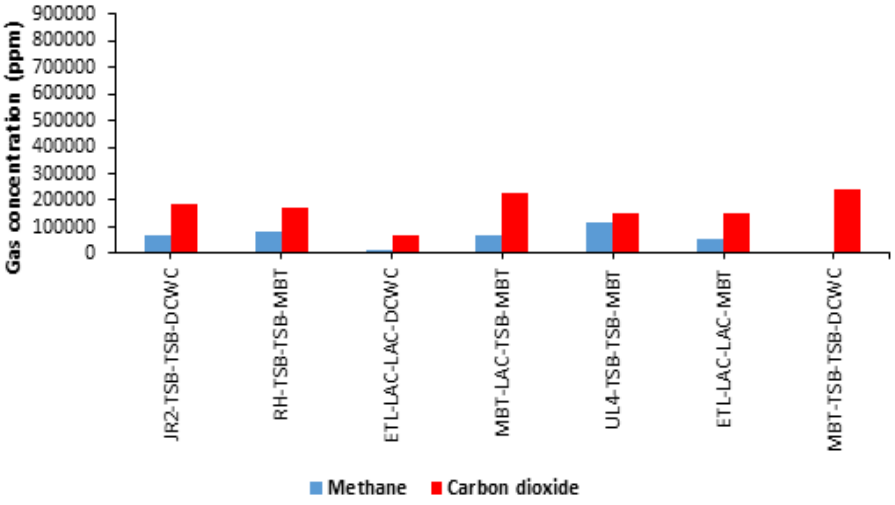
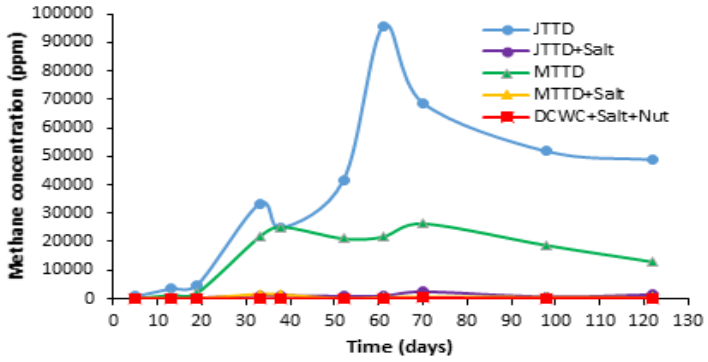
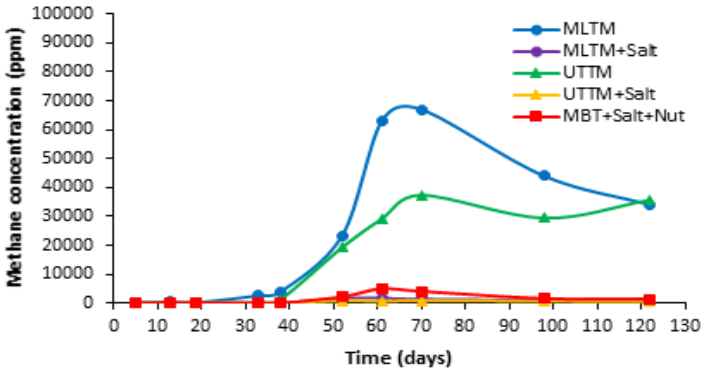


Figure 10. Gas production from microbial consortia in 100% v/v nutrient amendment

a)



b)



c)

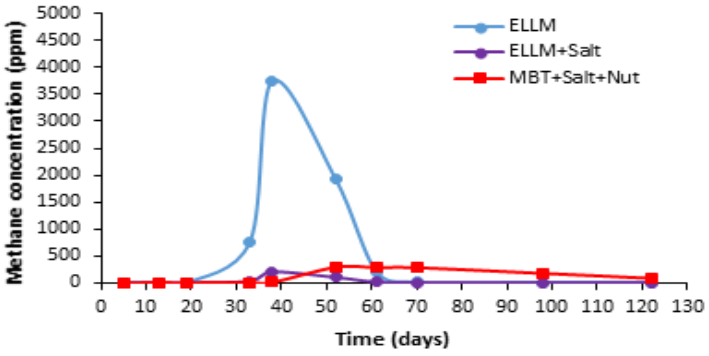


Figure 11. Methane production from microbial consortia with nutrient amendments (22.4% v/v) and [NaCl] 6.6 mg/cm^3 at 23°C. Blue and green colors depict microbial consortia (i.e., JTTD, MTTD, MLTM, UTTM, and ELLM) plus nutrient amendments, salt solutions and coal sources ("S1" in Table 12). Red color depicts coal sources (i.e., DCWC, MBT) plus nutrient amendments and salt solutions ("C1" in Table 12). Purple and orange colors depict microbial consortia plus salt solution ("C2" in Table 12).

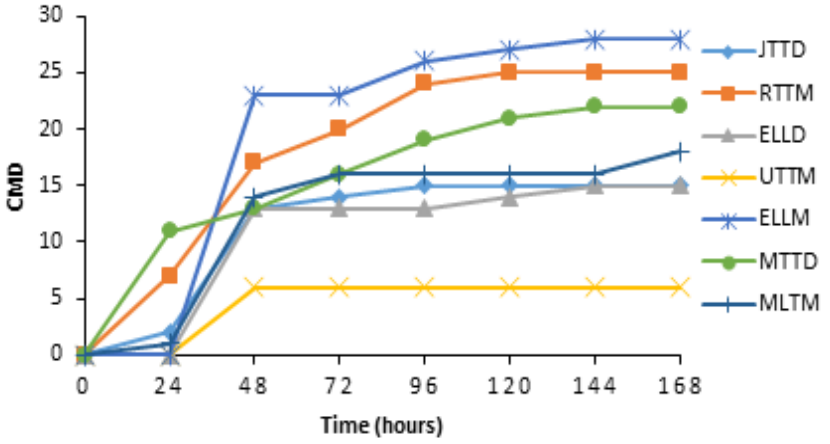


Figure 12. Community metabolic diversity

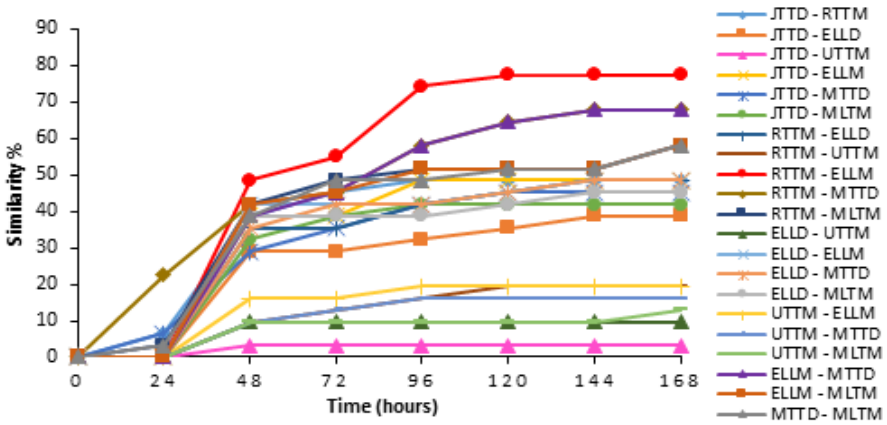


Figure 13. Percent similarity between microbial consortia

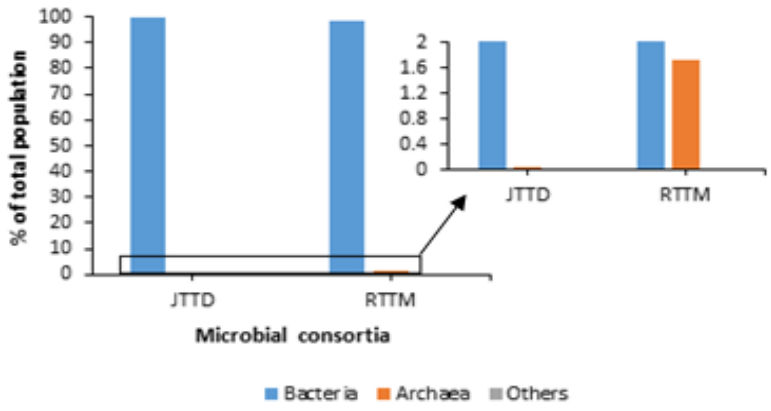


Figure 14. Distribution of different kingdoms for DNA extracted from microbial consortia

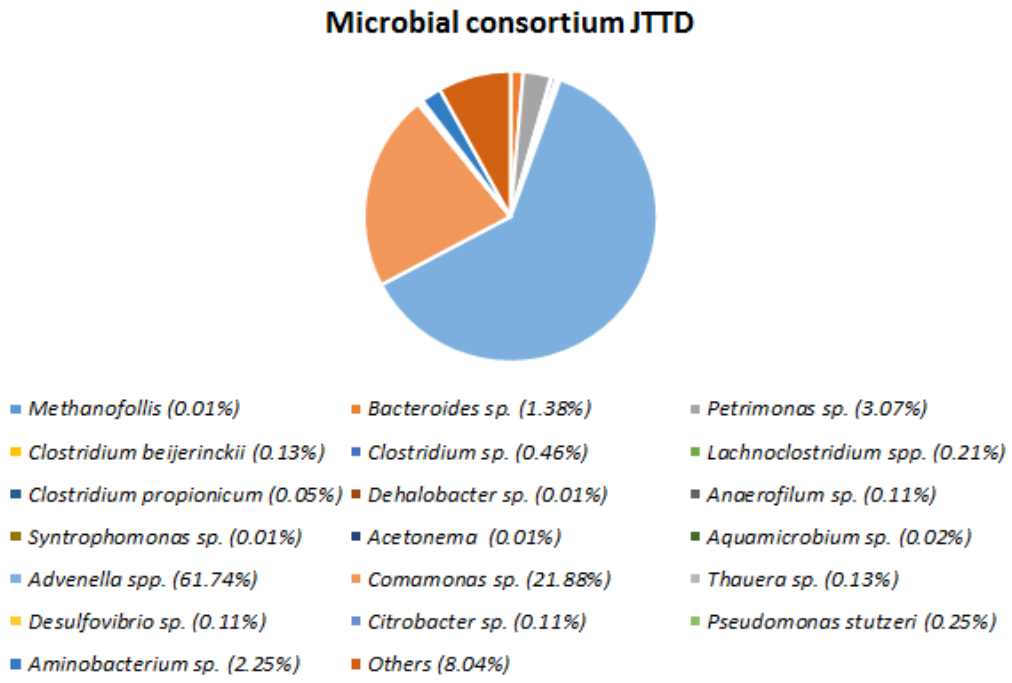


Figure 15. Diversity of microbes in the microbial consortium JTTD at 23°C

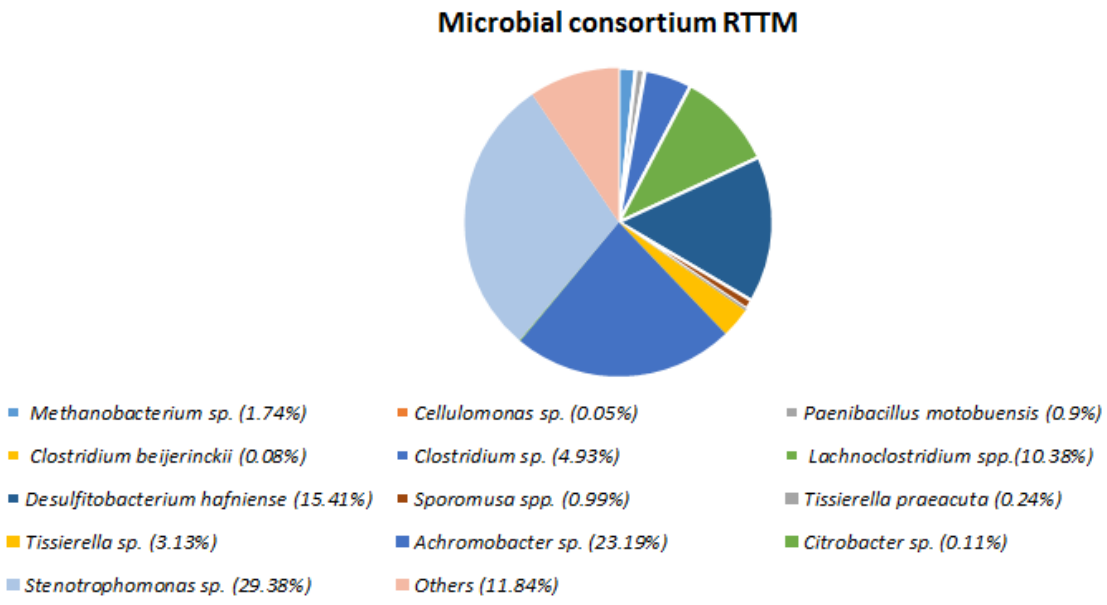


Figure 16. Diversity of microbes in the microbial consortium RTTM at 23°C

5.6. References

Adams, D.J., Opara, A.O., 2015. Optimization of biogenic methane production from hydrocarbon sources. U.S. Patent WO 2015013531 A1.

Ali-Shah, F., Mahmood, Q., Maroof-Shah, M., Pervez, A., Ahmad-Asad, S., 2014. Microbial ecology of anaerobic digesters: The key players of anaerobiosis. Scientific World J. Art. No. 183752. http://doi.org/10.1155/2014/183752.

Baena, S., Garcia, J.L., Cayol, J.L., Ollivier, B., 2015. *Aminobacterium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-5. doi:10.1002/9781118960608.gbm01253.

Bao, Y., Huang, H., He, D., Ju, Y., Qi, Y., 2016. Microbial enhancing coal-bed methane generation potential, constraints and mechanism – a mini-review. J. Nat. Gas Sci. Eng. 35, 68-78. https://doi.org/10.1016/j.jngse.2016.08.035.

Barra, A., Fajardo, C., Grenni, P., Ludovica, M., Amalfitano, S., Ciccoli, R., Martin, M., Gibello, A., 2009. The role of a groundwater bacterial community in the degradation of the herbicide terbuthylazine. FEMS Microbiol. Ecol. 71, 127-136. doi:10.1111/j.1574-6941.2009.00787.x.

Benson, H.J., 2002. Microbiological Applications: Laboratory Manual in General Microbiology, eighth ed. McGraw Hill, New York.

Boone, D. R., 2015. *Methanobacterium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-8. doi:10.1002/9781118960608.gbm00495.

Botheju, D., Bakke. R., 2011. Oxygen effects in anaerobic digestion: A review. Open Waste Manag. J. 4, 1-19. doi:10.2174/1876400201104010001.

Bum-Han, K., Fuertez, J., Stella, A., Nguyen, V., Szendrei, A., Mclennan, J.D., Sparks, T.D., 2017. Calcium alginate polymer encapsulation of proppant with time-release delivery of microbial consortia for methanogenesis. Energy Fuels. Submitted.

Buswell, E.G., Neave, S.L., 1930. Laboratory studies of sludge digestion. Illinois Division of State Water Survey, Bulletin No. 30. http://hdl.handle.net/2142/94553 (accessed 15.11.2016).

Busse, H.J., Auling, G., 2015. *Achromobacter*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-14. doi:10.1002/9781118960608.gbm00926.

Colosimo, F., Thomas, R., Lloyd, J.R., Taylor, K.G., Boothman, C., Smith, A.D., Lord, R., Kalin, R.M., 2016. Biogenic methane in shale gas and coal bed methane: A review

of current knowledge and gaps. Int. J. Coal Geol. 165, 106-120. https://doi.org/10.1016/j.coal.2016.08.011.

Del Real, J.O., 2007. Evaluation and modeling of anaerobic depuration kinetics from stillage of alcohol industry. Ph.D. Dissertation. Institute of Basic Sciences and Engineering, Autonomous University of Hidalgo State. Mexico.

Drake, H. L., Gössner, A. S., 2015. *Sporomusa*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-7. doi:10.1002/9781118960608.gbm00706.

Fallgren, P.H., Zeng, C., Ren, Z., Lu, A., Ren, S., Jin, S., 2013. Feasibility of microbial production of new natural gas from non-gas-producing lignite. Int. J. Coal Geol. 115, 79-84. https://doi.org/10.1016/j.coal.2013.03.003.

Ferry J.G., 1993. Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall, New York.

Frederiksen, W., 2015. *Citrobacter*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-23. doi:10.1002/9781118960608.gbm01143.

Fuchs, A., Lyautey, E., Montuelle, B., Casper, P., 2016. Effects of increasing temperatures on methane concentrations and methanogenesis during experimental incubation of sediments from oligotrophic and mesotrophic lakes. J. Geophys. Res. Biogeosci. 121, 1394-1406. doi:10.1002/2016JG003328.

Fuertez, J., Boakye, R., Mclennan, J.D., Adams, D.J., Sparks, T.D., Gottschalk, A., 2017. Developing methanogenic microbial consortia from diverse coal sources and environments. J. Nat. Gas Sci. Eng. In Press. doi:10.1016/j.jngse.2017.07.028.

Furmann, A., 2011. Microbial methanogenesis from coal extracts in experimental bioreactors. M.Sc. Dissertation. Department of Geological Sciences, Indiana University. U.S.

Furmann, A., Schimmelmann, A., Brassell, S.C., Mastalerz, M., Picardal, F., 2013. Chemical compounds classes supporting microbial methanogenesis in coal. Chem. Geol. 339, 226-241. https://doi.org/10.1016/j.chemgeo.2012.08.010.

Garland, J.L., 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. FEMS Microbiol. Ecol. 24 (4), 289-300. doi:10.1111/j.1574-6941.1997.tb00446.x.

Green, M.S., Flanegan, K.C., Gilcrease, P.C., 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76, 34-45. https://doi.org/10.1016/j.coal.2008.05.001.

Guo, H., Yu, Z., Thompson, I.P., Zhang, H., 2014. A contribution of hydrogenotrophic methanogenesis to the biogenic coal bed methane reserves of Southern Qinshui Basin, China. Appl. Microbiol. Biotechnol. 98, 9083-9093. doi:10.1007/s00253-014-5908-z.

Gupta, P., Gupta, A., 2014. Biogas production from coal via anaerobic fermentation. Fuel. 118, 238-242. https://doi.org/10.1016/j.fuel.2013.10.075.

Harris, S.H., Smith, R.L., Barker, Ch.E., 2008. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. Int. J. Coal Geol. 76, 46-51. https://doi.org/10.1016/j.coal.2008.05.019.

Head, I.M., Gray, N.D., Larter, S.R., 2014. Life in the slow lane: Biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front. Microbiol. 5, 1-23. doi:10.3389/fmicb.2014.00566.

Heider, J., Fuchs, G., 2015. *Thauera*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-11. doi:10.1002/9781118960608.gbm01004.

Holliger, C., 2015. *Dehalobacter*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-4. doi:10.1002/9781118960608.gbm00657.

Ibanez, J.G., Hernandez-Esparza, M., Doria-Serrano, C., Fregoso-Infante, A., Mohan, M., 2007. Environmental Chemistry: Fundamentals. Springer Science-Business Media, New York.

Jarrell, K.F., 1985. Extreme oxygen sensitivity in methanogenic Archaebacteria. Bioscience. 35 (5), 298-302. doi:10.2307/1309929.

Jones, E., Voytek, M., Corum, M., Orem W., 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76, 7013-7022. doi:10.1128/AEM.00728-10.

Kato, M.T., Field, J.A., Lettinga, G., 1997. Anaerobe tolerance to oxygen and the potentials of anaerobic and aerobic cocultures for wastewater treatment. Braz. J. Chem. Eng. 14 (4), 1678-4383. http://dx.doi.org/10.1590/S0104-66321997000400015.

Kayhanian, M., Tchobanoglous, G., Brown, R., 2007. Biomass conversion processes for energy recovery, in: Kreith, F., Goswami, D.G. (Eds.), Handbook of Energy Conservation and Renewable Energy, Taylor and Francis Group., Londres, pp. 67. https://doi.org/10.1201/9781420003482.ch25.

Kiener, A., Leisinger, T., 1983. Oxygen sensitivity of methanogenic bacteria. Syst. App.

Microbiol. 4 (3), 305-312. https://doi.org/10.1016/S0723-2020(83)80017-4.

Kirby, T., Lancaster, J., Fridovich, I., 1981. Isolation and characterization of the ion-containing superoxide dismutase of *Methanobacterium bryantii*. Arch. Biochem. Biophys. 210, 140-148.

Kuever, J., Rainey, F. A., Widdel, F., 2015. *Desulfovibrio*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-17. doi:10.1002/9781118960608.gbm01035.

Lavania, M., Cheema, S., Manab, P., Ganapathi, R., 2014. Methanogenic potential of a thermophilic consortium enriched from coal mine. Int. Biodeterior. Biodegradation. 93, 177-185. https://doi.org/10.1016/j.ibiod.2014.05.022.

Liu, Y., Boone, D.R., 1991. Effects of salinity on methanogenic decomposition. Bioresour. Technol. 35 (3), 271-273. https://doi.org/10.1016/0960-8524(91)90124-3.

Lupa, B. and Wiegel, J., 2015. *Desulfitobacterium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-13. doi:10.1002/9781118960608.gbm00658.

Megonigal, J.P., Hines, M.E., Visscher, P.T., 2013. Anaerobic metabolism: Linkages to trace gases and aerobic processes, in: Turekian, K., Holland, H. (Eds.), Treatise on Geochemistry, second ed. Elsevier Inc., Oxford, pp. 273-359. doi:10.1016/B978-0-08-095975-7.00808-1.

Meslé, M., Dromart, G., Oger, P., 2013. Microbial methanogenesis in subsurface oil and coal. Res Microbiol. 164 (9), 959-972. https://doi.org/10.1016/j.resmic.2013.07.004.

Massett, J., Calusinka, M., Hamilton, C., Hiligsmann, S., Joris, B., Wilmotte, A., Thonart, P., 2012. Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium spp*. Biotechnol. Biofuels. 5-35. doi:10.1186/1754-6834-5-35.

Nolla-Ardevol, V., Peces, M., Strous, M., Tegetmeyer, H., 2015. Metagenome from spirulina digesting biogas reactor: Analysis via binning of contigs and classification of short reads. BCM Microbiol. 15, 277. doi:10.1186/s12866-015-0615-1.

Opara, A., Adams, D.J., Free, M.L., Mclennan, J., Hamilton, J., 2012. Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste materials. Int. J. Coal Geol. 96-97, 1-8. https://doi.org/10.1016/j.coal.2012.02.010.

Opara, A., 2012. Biochemically enhanced methane production from coal. PhD. Dissertation. Department of Civil and Environmental Engineering, University of Utah. U.S.

Orem, W.H., Voytek, M.A., Jones, E.J., Lerch, H.E., Bates, A.L., Corum, M.D., 2010. Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. Org. Geochem. 41 (9), 997-1000. https://doi.org/10.1016/j.orggeochem.2010.03.005.

Palleroni, N. J., 2015a. *Pseudomonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1. doi:10.1002/9781118960608.gbm01210.

Palleroni, N. J., 2015b. *Stenotrophomonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-20. doi:10.1002/9781118960608.gbm01237.

Papendick, S.L., Downs, K.R., Vo, K.D., Hamilton, S.K., Dawson, G.K., Golding, S.D., 2011. Biogenic methane potential for Surat Basin, Queensland coal seams. Int. J. Coal Geol. 88, 123-134. https://doi.org/10.1016/j.coal.2011.09.005.

Park, Y.S., Liang, Y., 2016. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). Fuel. 166, 258-267. https://doi.org/10.1016/j.fuel.2015.10.121.

Preston-Mafham, J., Boddy, L., Randerson, P.F., 2002. Analysis of microbial community functional diversity using sole-carbon-source utilization profiles – a critique. FEMS Microbiol. Ecol. 42, 1-14. doi:10.1111/j.1574-6941.2002.tb00990.x.

Priest, F.G., 2015. *Paenibacillus*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-40. doi:10.1002/9781118960608.gbm00553.

Rainey, F.A., Hollen, B.J., Small, A.M., 2015. *Clostridium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-122. doi:10.1002/9781118960608. gbm00619.

Rainey, F.A., 2015a. *Lachnospiraceae fam. nov*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-2. doi:10.1002/9781118960608.fbm00133.

Rainey, F.A., 2015b. *Acetonema*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-5. doi:10.1002/9781118960608.gbm00686.

Rathi, R., Priya, A., Vohra, M., Lavania, M., Lal, B., Sarma, P., 2015. Development of a microbial process for methane generation from bituminous coal at thermophilic conditions. Int. J. Coal Geol. 147-148, 25-34. https://doi.org/10.1016/j.coal.2015.06.004.

Ritter, D., Vinson, D., Barnhart, E., Akob, D., Fields, M., Cunningham, A., Orem, W., McIntosh, J., 2015. Enhanced microbial coalbed methane generation: A review of research, commercial activity, and remaining challenges. Int. J. Coal Geol. 146, 28-41. https://doi.org/10.1016/j.coal.2015.04.013.

Schnürer, A., Jarvis, A., 2010. Microbiological handbook for biogas plants. Swedish Waste Management U2009:03. Swedish Gas Centre Report 207. http://www.eac-quality.net/fileadmin/eac_quality/user_documents/3_pdf/Microbiological_handbook_for_biogas_plants.pdf (accessed 25.2.2017).

Sekiguchi, Y., 2015. *Syntrophomonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-11. doi:10.1002/9781118960608.gbm00682.

Shah, H.N., Hookey, J.V., 2015. *Tissierella*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-3. doi:10.1002/9781118960608.gbm00721.

Shen, C.F., Guiot, S.R., 1996. Long term impact of dissolved O_2 on the activity of anaerobic granules. Biotechnol. Bioeng. 49 (6), 611-620. doi:10.1002/(SICI)1097-0290(19960320)49:6<611::AID-BIT2>3.0.CO;2-R.

Stackebrandt, E., Schumann, P., 2015. *Cellulomonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-14. doi:10.1002/9781118960608.gbm00063.

Steubing, P.M., 1993. Isolation of an unknown bacterium from soil, in: Tested Studies for Laboratory Teaching. Proceedings of the 14th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). Las Vegas, U.S. June 2nd-6th. https://eric.ed.gov/?id=ED395809 (accessed 15.2.2016).

Villemur, R., Lanthier, M., Beaudet, R., Lepine, F., 2006. The *Desulfitobacterium* genus. FEMS Microbiol. Rev. 30, 706-733. doi:10.1111/j.1574-6976.2006.00029.x.

Wawrik, B., Mendivelso, M., Parisi, V.A., Suflita, J.M., Davidova, I.A., Marks, C.R., 2012. Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. FEMS Microbiol. Ecol. 81, 26-42. doi:10.1111/j.1574-6941.2011.01272.x.

Willems, A., Gillis, M., 2015. *Comamonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1–17. doi:10.1002/9781118960608.gbm00945.

Wolfe, R.S., 2011. Techniques for cultivating methanogens. Methods Enzymol. 494, 1-22. doi:10.1016/B978-0-12-385112-3.00001-9.

Wu, S., Bhattacharjee, A.S., Weissbrodt, D.G., Morgenroth, E., Goel, R., 2016. Effect of short term external perturbations on bacterial ecology and activities in a partial nitritation and anammox reactor. Bioresour. Technol. 219, 527-535. https://doi.org /10.1016/j.biortech.2016.07.118.

Wu, Z.G., Wang, F., Gu, C.G., Zhang, Y.P., Yang, Z.Z., Wu, X.W., Jiang, X., 2014. *Aquamicrobium terrae sp*. Nov., Isolated from polluted soil near a chemical factory. Antonic Van Leeuwnhock. 105 (6), 1131-1137. doi:10.1007/s10482-014-0174-8.

Yutin, N., Galperin, M., 2013. A Genomic update on clostridial phylogeny: Gram-negative spore-formers and other misplaced Clostridia. Environ. Microbiol. 15 (10), 2631-2641. doi:10.1111/1462-2920.12173.

Zak, J.C., Willig, M.R., Moorhead, D.L., Wildman, H.G., 1994. Functional diversity of microbial communities: A quantitative approach. Soil Biol. Biochem. 26 (9), 1101-1108. https://doi.org/10.1016/0038-0717(94)90131-7.

Zellner, G., Boone, D.R., 2015. *Methanofollis*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-7. doi:10.1002/9781118960608.gbm00506.

Zhang, J., Liang, Y., Pandey, R., Harpalani, S., 2015. Characterizing microbial communities dedicated for conversion of coal to methane in situ and ex situ. Int. J. Coal Geol.146, 145-154. https://doi.org/10.1016/j.coal.2015.05.001.

Zhang, J., Park, S., Liang, Y., Harpalani, S., 2016. Finding cost-effective nutrient solutions and evaluating environmental conditions for biogasifying bituminous coal to methane ex situ. Appl. Energy. 165, 559-568. https://doi.org/10.1016/j.apenergy. 2015.12.067.

Zupančič, G.D., Grilc, V., 2012. Anaerobic treatment and biogas production from organic waste, in: Kumar, S., Bharti, A. (Eds.), Management of Organic Waste. InTech., Rijeka, pp. 1-28. doi:10.5772/32756.

6. OPTIMIZATION OF BIOGENIC METHANE PRODUCTION FROM COAL

6.1. Abstract

Given continuously increasing global energy needs, diversified efforts have been made to find and exploit new natural gas resources. These include coalbed methane (CBM), which represents an important global, unconventional source of natural gas. Efforts have been underway for some time to more effectively generate methane in situ in coal plays by introduction of nutrients and/or microbial consortia. However, much is still to be learned about the limitations and environmental conditions that support microbial growth and are conducive to biogenic methane production from coal. The present investigation evaluated environmental conditions that led to increased methane production from subbituminous coal by introducing a methanogenic consortium that included *Methanobacterium sp.* A central composite design (CCD) was used to explore a broad range of operational conditions, examine the effects of the important environmental factors, such as temperature, pH and salt concentration, and query a feasible region of operation to maximize methane production from coal. An anticipated detrimental effect of NaCl concentration on methane production was observed for the consortium assessed. The range of feasible operational conditions comprised initial pH values between 4.2 and 6.8, temperatures between 23°C and 37°C, and NaCl concentrations between 3.7 mg/cm^3 and 9.0 mg/cm^3. Coal biogasification was optimal for this consortium at an initial pH value of

5.5, at 30°C, and at a NaCl concentration 3.7 mg/cm^3 (i.e., 145,165 ppm, which is 25.6 sft^3/ton).

6.2. Introduction

Coal is a major source of energy worldwide (Rathi et al., 2015). As an abundant and inexpensive resource, it has been investigated extensively for generating fuels and chemicals through different conversion technologies, including traditional combustion for power generation (Zhang et al., 2016a). Recently, biogasification or bioconversion of coal has emerged as an important alternative with significant economic benefits. Even more important is the opportunity to use coal in situ in a more environmental fashion (Bao et al., 2016; Wolkein et al., 1994).

Biogasification using microorganisms converts coal into methane, and can be used for both in situ (abandoned or unmineable coal seams) and ex situ (coal waste accumulations near coal mines) applications (Zhang et al., 2016a). Substantial efforts have been dedicated to making coal biogasification commercially viable with microbially enhanced coalbed methane protocols (Park and Liang, 2016; Ritter et al., 2015; Zhang et al., 2016a). However, research in this area is still required and a great deal must still be learned about the limitations to microbial growth and biogenic methane production in coalbeds (Bao et al., 2016; Budwill, 2003).

A growing interest for optimizing methane production using foreign or indigenous microbes has recently emerged (Adams and Opara, 2015; Downey, 2010; Green et al., 2008; Guo et al., 2014; Zhang et al., 2016a). Bioaugmentation with select microbial consortia and stimulation of indigenous microbes with added nutrients have shown their

potential to produce methane from coal and increase gas productivity during short-term engineering operations (Park and Liang, 2016; Ritter et al., 2015).

Recent studies (Green et al., 2008; Gupta and Gupta, 2014; Lavania et al., 2014; Rathi et al., 2015; Zhang et al., 2016a) have used simple optimization strategies to determine maximum methane production and identify optimal operational conditions in laboratory settings. Some parameters have been optimized for a given microbial community with select coal ranks. Among these strategies, a simple evaluation, with methane production as the dependent variable, has been carried out. While one parameter is changed, others parameters are kept constant. This technique is commonly called "one-variable-at-a-time," and its major disadvantage is that the interactive effects among variables cannot be determined (Almeida et al., 2008; Zhang et al., 2016a). Hence, the complete effect of the evaluated parameters on the response have not been assessed.

Recognizing that an evaluation of biogenic methane production from coal is mostly conducted under strict anaerobic conditions (Beckmann et al., 2011; Green et al., 2008; Gupta and Gupta, 2014; Harris et al., 2008; Jones et al., 2010; Lavania et al., 2014; Orem et al., 2010; Papendick et al., 2011; Rathi et al., 2015; Wawrik et al., 2012), the use of complex optimization approaches would be less laborious and cost-intensive, allowing examination of mutual interactions between factors with a reduced number of experiments. The identification of optimal operating conditions and description of relevant factors can be important elements for achieving the best system performance and optimizing gas production (Park and Liang, 2016; Zhang et al., 2016a).

To date, published studies have not evaluated controlling factors for maximized methane production from coal when using a methanogenic consortium developed under

initial atmospheric exposure. This aero-tolerance is a key consideration for use in field-scale operations. The aim of this study is to use an experimental design to broadly explore a range of operational conditions and examine the main factors. These factors include temperature, pH, and salinity (NaCl concentration). In addition, this defined a feasible region of functionality that includes the maximum methane production from a particular subbituminous coal. The results are expected to lead to an in-depth investigation of alternative strategies to optimize coal biogasification.

6.3. Materials and Methods

6.3.1. Coal Sources

Lignitic Red Hills coal – RH was previously used as source of microbial populations during the development of microbial consortium (Fuertez et al., 2017); subbituminous Miller Black Thunder - MBT coal was used as a substrate in this study. Proximate and ultimate analyses are shown in Table 18. The experimental program used six gram aliquots of pulverized Miller Black Thunder – MBT coal (-140 mesh). These were placed in 50 mL-sterile conical tubes. The conical tubes were used as bioreactors (Adams and Opara, 2015; Fuertez et al., 2017). Arguably, the particle size of the coal can have a significant effect on the biogenic methane production (Bao et al., 2016; Green et al., 2008; Gupta and Gupta, 2014), and the results presented herein may represent a possible and desired scenario. The coal samples were exposed to air during storage, handling and preparation, which may have also influenced their biodegradability and bioavailability.

6.3.2. Inoculum Preparation

The methanogenic consortium used in this study was developed using a three-phase screening program, as described in Fuertez et al. (2017). Briefly, microbial samples were initially collected from various hydrocarbon-rich environments and locations characterized by natural methanogenesis. Three screening phases were implemented to select promising microbial samples that produced large amounts of methane under initial atmospheric exposure. Enrichment with favorable nutrient amendments as well as a final adaptation step to selected coal sources were carried out. Thus, microbial populations within the chosen consortium comprised indigenous microbial populations from Red Hills lignite – RH enriched with tryptic soy broth – TSB and adapted to subbituminous Miller Black Thunder – MBT coal for approximately twenty-four weeks. The described experimental program was used to label this consortium as RTTM (Fuertez et al., 2017). These adapted microbes were separated from the enriched culture and were maintained in 15 g/L tryptic soy broth (Sigma-Aldrich®) at 30°C for twenty-five days (Lavania et al., 2014). Aseptic microbiological techniques were used for all procedures reported herein (Opara, 2012).

Prior to inoculation of the bioreactors, the microbial consortium was washed with a sterile saline solution - 8.5 mg/cm^3 NaCl - to remove any remaining nutrient media. An initial cell concentration of 2.0 x 10^7 cfu/mL was determined by colony counting on TSA plates. Aerobic, aerotolerant and culturable organisms were particularly counted (Benson, 2002; Opara, 2012). An aliquot of this culture was used for DNA extraction. DNA was extracted and purified using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA) in accordance with the manufacturer's protocols. Subsequently, analysis and identification of microbial communities were performed using a 16S rRNA gene-based amplicon

sequencing at the Research and Testing Laboratory (Lubbock, TX, USA). A 97% of similarity was reported for identification of microbial populations (Wu et al., 2016).

6.3.3. Media Preparation for Bioreactors

Four milliliters of inoculum, 8.4 mL of stock nutrient solution (15 g/L, TSB), and 12.6 mL of sterile sodium chloride solution were added into individual 50 mL-bioreactors each containing 6 g of subbituminous Miller Black Thunder – MBT coal. An approximate free headspace of 17.5 mL was obtained. Duplicate samples were prepared and simultaneously evaluated with two control types. Type I control samples included NaCl solution plus coal material. It is hypothesized that gases measured from these controls corresponded to residual gas desorption and/or generation from indigenous microbes (Fuertez et al., 2017; Opara, 2012; Opara et al., 2012). Type II control samples included the microbial consortium plus the nutrient and the NaCl solution. These controls were used to consider gas production by the microbial consortium from the added nutrients.

The concentration of the sodium chloride solution was fixed according to the design of experiments (Table 19): a sodium ion selective electrode (Thomas Scientific® model 3401BN) attached to a benchtop multiparameter meter (Thermo Scientific Orion, VSTAR00 VERSA STAR^{MT}) (Garcia et al., 1991) was initially used to estimate sodium concentration in coal, nutrient amendment, and inoculum. Before inoculation, pH levels of the liquid solutions (i.e., nutrient plus NaCl solutions) and the coal samples were established. Subsequently, pH levels were double checked after inoculation with a sterilized and continuously calibrated pH soil probe (PROBSOIL, Bluelab Corporation Limited- New Zealand). Solutions of hydrochloric acid 2.4N and potassium hydroxide

5.0N were prepared to adjust pH levels (Rathi et al., 2015).

The bioreactors were set aside without agitation during incubation. Unless otherwise specified, gas measurements were conducted every fifteen days for two and a half months in order to identify overall trends of methane production. Gas chromatography was used for this purpose. Considering that effective mesophilic methanogens can have longer regeneration times and slower growth than other microorganisms involved in the biotransformation of complex organic matter, long periods of consortia incubation were initially evaluated (Del Real, 2007; Ferry, 1993; Zupančič and Grilc, 2012).

After the prescribed periods of reaction, two-hundred microliters (200 µL) of produced gas were directly extracted under sterile conditions using a gas-tight syringe (Hamilton 1725, Hamilton Robotics, Reno, NV) through a small hole in the cap that had been completely covered with silicone gel. Silicone was periodically reapplied to the end caps to prevent leakage (Bao et al., 2016; Fallgren et al., 2013). In addition to this precautionary measure, the bioreactors can be visually checked by spreading a solution of gas leak detector and checked for bubble generation (ASTM E515-05).

To allow the microbial communities to evolve under initial atmospheric conditions that would be similar to those during their development, the bioreactor headspace was not sparged with nitrogen gas and reducing agents (e.g., Na_2S, cysteine-HCL) were not used (Fuertez et al., 2017). These initial conditions were intentionally chosen. This demonstrated the consortium's ability to survive and still produce methane at low to moderate oxygen concentrations during the experimental program. This can be an important consideration for use in field-scale operations where oxygen exposure is anticipated.

6.3.4. Parameter Selection

Based on preliminary studies of aerotolerant methanogenic consortia, the main factors that significantly affect microbial growth and methane production from coal are temperature, salt concentration, and pH (Opara, 2012). Additionally, these variables are among the main regulating factors of methanogenesis. Their influence and relevance to methane production from complex organic matter has been examined by various researchers (Green et al., 2008; Gupta and Gupta, 2014; Hoehler et al., 2010; Liang, 2015; Megonigal, et al., 2013; Rathi et al., 2015). Considering the importance of these selected factors on coal biogasification, a parametric space that includes maximum methane production was experimentally defined. A broad range of operational conditions was initially chosen:

- NaCl concentrations were varied from 3.7 mg/cm^3 to 74.3 mg/cm^3,
- pH values of the culture medium ranged between 4.2 and 10.2, and
- Temperatures were varied from 23°C to 54°C.

These ranges were inferred from the literature as possible conditions for methanogenic activity (Ferry, 1993; Goodwin et al., 1987; Green et al., 2008; Gupta and Gupta, 2014; Hedderich and Whitman, 2006; Jones et al., 2010; Kim et al., 2004; Lavania et al., 2014; Liu and Boone, 1991; Lu et al., 2015; Nozhevnikova et al., 2007; Opara, 2012; Opara et al., 2012; Orem, 2010; Papendick et al., 2011; Waldron et al., 2007; Zhang et al., 2015).

6.3.5. Central Composite Design (CCD)

To determine a region of operation and broadly explore the effects of temperature, pH and NaCl concentration, a central composite design (CCD) was used (Almeida et al., 2008; Khuri and Mukhopadhyay, 2010; Myers and Montgomery, 1995). Gas concentrations were expressed as percentage of the maximum experimental methane concentration (76,000 ppm). This percent concentration was defined as the response variable – Y_{CH4} to facilitate computational analysis. Preliminary studies have shown nonlinear response of methane production when these important environmental factors were examined (Green et al., 2008; Gupta and Gupta, 2014; Liang, 2015; Opara, 2012; Rathi et al., 2015; Zhang et al., 2016a). Thus, a quadratic model was adopted as an initial approach to describe the relationships between these selected factors and methane production. This nonlinear mathematical model is known to be a flexible and simple model that can take multiple functional forms (Myers and Montgomery, 1995).

The central composite design contained a factorial set of 2^3 experiments with 3 center points and 6 axial points for estimation of curvature. These axial points established extrema for the low and high settings for all factors. A total of 17 experiments were defined (Table 19). The number of experimental runs at center point, and the distance of axial points ($\alpha = 1.682$) were chosen according to a rotatable design (Almeida et al., 2008; Khuri and Mukhopadhyay, 2010; Myers and Montgomery, 1995). CCD has a spherical symmetry and requires 5 levels for each factor. This allows exploring a large process space with a reduced number of experiments. The three factors describe a sphere around a factorial cube determined by the factorial set (Almeida et al., 2008; Myers and Montgomery, 1995). This experimental design provides the highest quality of predictions over the entire design space

(Khuri and Mukhopadhyay, 2010; Myers and Montgomery, 1995).

The statistics software package STATGRAPHICS Centurion VII® was used to create the experimental design, perform regression analysis of the data obtained, and to estimate the coefficients of the model equations. Additionally, MatLab® was used for complementary graphical analysis. The analysis of variance ANOVA was conducted in order to obtain interaction of the process variables with the response variable. The overall predictive capability of the model equations was expressed by the coefficient of determination - R^2 (Almeida et al., 2008; Elaiyaraju and Partha, 2016).

6.3.6. Determination of a Region of Operation

A feasible region of operation for the selected factors was sought. Methane concentrations were measured over time and analyzed with STATGRAPHICS Centurion VII®. This captured the main trends of gas production and identified important effects of the factors evaluated. Empirical models were generated at selected times. MatLab® was used as a complementary tool to visualize the predictions from the regression equations using a three-dimensional graph (i.e., scatter3 – 3D scatter plot), and to graphically estimate a feasible region of operation: model predictions were stored and organized from the smallest to the largest value. This procedure accounted for the levels of the factors and the time when gas measurements were made. The obtained region of operation included model predictions for up to sixty days of incubation – the period of time where the largest methane production was measured and the best-fitted regression equations were found.

6.3.7. Verification of Methane Production from the Predicted Region of Operation

To verify the predicted sweet spot for methane production, additional experiments were conducted. Three 50 mL-bioreactors were used. The main bioreactor contained six grams of subbituminous Miller Black Thunder coal - MBT, 8.4 mL of stock nutrient solution (15 g/L, TSB), 4 mL of inoculum with an initial cell concentration of 2.5×10^6 cfu/mL, and 12.6 mL of sterile NaCl solution. An approximate free headspace of 17.5 mL was obtained. The salt concentration and pH were fixed at 3.7 mg/cm^3 and 5.5, respectively. Two control samples were also used under the same environmental conditions: The Type I control sample included NaCl solution plus coal material, and the Type II control sample included microbial consortium plus nutrient and NaCl solution. These bioreactors were kept at 30°C. They were not agitated and were monitored over time for headspace methane production.

6.3.8. Gas Measurements

Methane concentrations (ppm) were determined with a Hewlett Packard HP6890 GC system (Palo Alto, CA) with a GS-GasPro PLOT column containing a bonded, silica-based stationary phase. These measurements were conducted regularly as indicated in the section Media Preparation for Bioreactors in Chapter 6. A flame ionization detector (FID) and thermal conductivity detector (TCD) were connected to analyze organic compounds and inorganic gases, respectively. Helium was the gas carrier. The temperature program began at 35°C for 4 min to allow methane elution and the temperature was then increased by 25°C min^{-1} to 260°C. Scotty Analyzed Gases were used as standards to build calibration curves. GC ChemStation (Agilent Technologies) computer software was used.

6.4. Results and Discussion

A central compose design (CCD) was employed to explore a broad range of operational conditions, and examine the effects of temperature, pH, and salt concentration. The experimental procedures identified a feasible region of operation that includes the maximum methane production from a particular subbituminous coal exposed to a methanogenic microbial consortium. A small set of experiments was used to systematically vary selected factors. Subsequent analysis of the experimental data identified those factors that most influence the results and the interactive effects among variables. The empirical models relating environmental factors to the response variable enabled an overall view of the system behavior. Model predictions were confirmed with a validation experiment.

6.4.1. Microbial Community Present in the Culture

The diversity assay revealed that the methanogenic microbial consortium contained 96.3% bacterial strains and 3.7% archaea. Similar percentages of total population for enriched and adapted consortia on coal can be found in the literature (Meslé et al., 2013; Zhang et al., 2015). The microbial consortium used for this study was separated from the initial enriched culture, and was maintained in fresh nutrient media (Lavania et al., 2014; Rathi et al., 2015). Thus, possible changes of microbial communities could have occurred (Green et al., 2008).

Nineteen bacterial species and one species of archaea comprised the microbial community. The most abundant bacterial species were (Figure 17): *Rhodococcus equi* which correspond to an aerobic or facultative anaerobic organism able to degrade poorly water-soluble organic compounds (Bell et al., 1998; Meslé et al., 2013; Stackebrandt and

Schumann, 2015). On the other hand, *Rummeliibacillus* spp., *Clostridium beijerinckii*, *Clostridium sp., Lachnoclostridium* spp., *Clostridium propionicum, Desulfitobacterium hafniense, Clostridium sporosphaeroides,* and *Tissierella sp.* included obligate and facultative anaerobes. These microbes comprised fermenters and syntrophs able to hydrolyze water-soluble macromolecular compounds, fatty acid oxidizers and acetogens (Meslé et al., 2013; Rainey et al., 2015; Shah and Hookey, 2015). The archaea were dominated by the *Methanobacteriales* order: *Methanobacterium sp.,* which is known as a hydrogenotrophic methanogen (Boone, 2015; Meslé et al., 2013). These identified microorganisms comprised metabolic groups commonly observed in the bioconversion of coal (Colosimo et al., 2016; Meslé et al., 2013; Zhang et al., 2015).

6.4.2. Experimental Design and Analysis of Variance - ANOVA

As mentioned, experimental runs were performed in duplicate. Data points were the average of the duplicate ± standard deviation (less than 25% of average). These were expressed as methane percent concentration and analyzed with STATGRAPHICS Centurion VII®. There was not significant methane production from the control samples. ANOVA results for the generated model after sixty days of incubation are represented in Table 20. Quadratic models and their coefficients of determination - R^2 are given in Table 21. All factors and their interactions were kept to show the initial structure of the regression models and to achieve the best fit to experimental data (Meyers and Montgomery, 1995).

The ANOVA table partitions the variability in Y_{CH4} into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. Table 20 shows the analysis of

variance after sixty days. In this table, one parameter (NaCl concentration) had a statistically significant effect with a p-value less than 0.05. These results indicated that the NaCl concentration was the most significant factor controlling methane production in these experiments. The combined interaction of NaCl concentration with itself (i.e., BB in Table 20) had a statistically significant effect in comparison to the combinations of other parameters. As for the effects of temperature and pH, p-values demonstrated that pH was more significant statistically than temperature. Similar behavior was observed for incubation times other than sixty days.

The coefficient of determination - R^2 varied between 0.58 and 0.71 for methane production, depending on the incubation time (Table 21). This statistical assessment provides a measure of how well the regression approximates the real data points, or how much variability in the observed response values can be explained by the experimental factors and their selected parameter interactions (Elaiyaraju and Partha, 2016). Thus, the regression models can explain a percentage up to 71 of the variability in methane production. A good fitted model should be characterized by a high R^2 value (> 0.5) (Elaiyaraju and Partha, 2016; Fabiszewska et al., 2015). Additionally, a plot of the residuals versus the predicted values of Y_{CH4} with the regression model obtained after sixty days of incubation is shown in Figure 18. This plot appears satisfactory to broadly explore an experimental region of operation and to evaluate the effects of the selected factors (i.e., temperature, pH, and salt concentration) on methane production (Meyers and Montgomery, 1995).

The generated models provided satisfactory prognostic capabilities. It is seen that high salt concentration would be detrimental to methane production. In addition, factors

such as particle size, coal loading (Bao et al., 2016; Green et al., 2008; Gupta and Gupta, 2014; Rathi et al., 2015), and other unknown factors can also influence the biodegradation of coal and methane production (Bao et al., 2016). Even though additional factors can affect the response values, the key factors considered here have mainly been evaluated by variation of single parameters using a one-variable-at-a-time technique (Green et al., 2008; Gupta and Gupta, 2014; Levania et al., 2014; Rathi et al., 2015; Zhang et al., 2016a).

6.4.3. Graphical Presentation of the Model Equations

3D surface plots for the effect of temperature and pH on coal biogasification, the effect of salt concentration and temperature, and the effect of salt concentration and pH are shown in Figures 19a through 19c, respectively. Figure 19a shows that at high temperature (54°C), less methane is produced. The largest gas production is in the vicinity of 30°C. Similarly, at high values of pH (> 7.2) less methane is produced. The largest volumes of methane are generated at low pH values (< 7.2) and low temperatures (< 39°C). Figure 19b shows that less gas is produced at elevated NaCl concentrations. A detrimental effect of salt concentration on methane production can be observed. Large amounts of methane are produced at the lowest salt concentration (3.7 mg/cm^3) and at low temperatures (< 39°C).

Figure 19c again shows the prominent effect of salt concentration. The largest methane production is obtained at the lowest NaCl concentration and at low pH values (< 7.2). It is likely that salt concentrations larger than 18 mg/cm^3 created lysis pressure that broke directly the membrane of cells, ceasing cellular metabolism and affecting gas production (Schnürer and Jarvis, 2010). Salt concentrations less than 18 mg/cm^3, however, might be suitable for methanogenesis. In addition, the curvature of the response surfaces

above 50 mg/cm^3 of salt concentration (Figure 19b – c) is likely related to the model that is fitted and not to the true behavior of the system (Meyers and Montgomery, 1995). Similar trends were observed for the other incubation periods.

After seventy-five days, methane levels dropped significantly in some bioreactors (e.g., #4 and #14 in Table 19). This scenario of methane content increasing and subsequently decreasing has been reported previously (Papendick et al., 2011; Zhang et al., 2015, 2016a, 2016b), suggesting further investigation is relevant. As a possible explanation, microbial methane oxidation could have occurred (Fuchs et al., 2016; Megonigal et al., 2013). Headspace gas was not completely removed or replaced during sampling for gas measurements; the bioreactors operated as a closed system. It is expected that methane concentration stabilizes at some point (Opara, 2012). Coal biogasification proceeded without any external intervention to influence gas production. It is also likely that self-inhibitory byproducts may have been present (Wang et al., 2017), and/or essential nutrients or trace elements may have depleted over time. This would decrease the microbial populations and consequently affect methane production. Finally, it is speculated that insufficient electron donors (i.e., H_2, acetate, formate) were available or were not produced continuously to support methanogenesis under the conditions considered.

6.4.4. Evaluation of Main Effects on Methane Production

An important characteristic of the interactions between microorganisms and their surroundings is the regulation of activities in response to environmental stimuli (Ferry, 1993). The role of temperature, salinity, and pH deserves further discussion.

6.4.4.1. Temperature

Methanogenic microbes can grow in a variety of temperature domains. In marine sediments at 2ºC to geothermal areas above 100°C. There is a great diversity of mesophilic and thermophilic species (Bergey and Holt, 1994; Ferry, 1993). Most of the mesophilic methanogens grow optimally at temperatures between 30°C and 37°C while thermophiles generally grow at temperatures between 50°C and 65°C, (Bergey and Holt, 1994; Ferry, 1993; Megonigal et al., 2013). Methanogenesis is often more affected by temperature than other biological processes (Megonigal et al., 2013). Microbial selection protocols for these experimental would have selected microbes that exhibited optimal methanogenesis at temperatures greater than 37°C. Different microbial screening/selection protocols could be used to select for microbial populations having optimal performance at higher temperatures and/or other conditions.

Many mesophiles prefer temperatures greater than ambient atmospheric temperature (Megonigal et al., 2013; Opara, 2012; Ritter et al., 2015). This has been observed with the microbial community used in this study. The temperature of 48°C might be considered to be the limit of tolerance since the microbial consortium was able to produce significant amounts of methane up to this value. These data suggest the possible use of the consortium for enhanced biogenic methane generation at higher temperatures than 30-35°C. Elevated temperatures are often observed in coal mines at great depth (Rathi et al., 2015). Assuming a mean surface temperature of 10°C and an average temperature gradient with depth of 1.8°C/100m, microbial activity using the consortium developed may cease below 2,665 m (8,743 ft) (Seidle, 2011).

Overall, between 23°C and 35°C, methane production can increase with

temperature, and to decrease at the highest value used in the experimental program, 54°C. This trend is in agreement with performance of a mesophilic microbial community comprised by the archaea *Methanosarcina mazei,* which had been isolated from mine water in Jitpur, India. Using coal from this mine, incubation was characterized by an increasing trend of methane production from 25°C to 35°C followed by a decreasing trend from 35°C to 55°C (Gupta and Gupta, 2014). Also, for a similar microbial consortium from the Fort Union Formation in the Powder River Basin, Wyoming, methane production from subbituminous Wyodak coal increased substantially after increasing the incubation temperature from 22°C to 38°C (Green et al., 2008).

Zhang et al. (2016a) reported increasing methane production between 24°C and 32°C during bioconversion of bituminous coal. Production was impaired at 40°C. This microbial community had been obtained from formation water in the Illinois basin, in the United States. Rathi et al. (2015) investigated methane production from bituminous coal from the Banaskantha coal mine in India, using a thermophilic methanogenic consortium. This consortium was obtained from formation water from the same mine. During incubation, methane production tended to increase from 37°C to 60°C and declined from 60°C to 70°C. Lavania et al. (2014) reported a similar trend for subbituminous coal and a thermophilic microbial consortium enriched from formation water from the Jharia coal mine in India. That consortium showed increasing methane production as the temperature changed from 37°C to 65°C; above 70°C a decline was observed.

Increasing temperature can enhance cell metabolism and growth kinetics. In addition, the solubility of coal substrates can be increased which in turn increases the rate and extent of substrate mass transfer from coal solids (Green et al., 2008; Lavania et al.,

2014; Rathi et al., 2015; Zhang et al., 2016a). However, if a threshold temperature is exceeded, certain microbes may be negatively affected (Zhang et al., 2016a). Above this threshold temperature, growth rate decreases and microbial activity may cease (Shuler and Kargi, 2002). Green et al. (2008) argued that if dissolution represents the rate-limiting step in methane production from coal solids, increased solubility will lead to enhanced methanogenesis.

6.4.4.2. Salinity

Methanogenic populations can survive in a wide range of salinities; from freshwater to hypersaline environments (Ferry, 1993). This type of salinity variation can be also found in petroleum reservoir formation waters (Head et al., 2014). Freshwater methanogens generally need at least 2.3 x 10^{-2} mg/cm^3 of sodium for their growth and metabolic functions (Ferry, 1993; Megonigal, et al., 2013; Patel and Roth, 1977). Hydrogenotrophic and acetoclastic methanogens are common in subsurface settings and are generally limited to lower-salinity conditions (Waldron et al., 2007). *Methanocalculus halotolerans* is the most halotolerant hydrogenotrophic methanogen reported; able to survive NaCl concentrations up to 120 mg/cm^3. This organism had been isolated from an oilfield brine (Head et al., 2014; Waldron et al., 2007).

Generally, methanogens are the organisms that are most affected by increasing salt concentrations (Patel and Roth, 1977; Schnürer and Jarvis, 2010). While salt can have an inhibitory effect, the concentration at which this is evident might vary depending on the substrate type and its availability (Schnürer and Jarvis, 2010). Typically, acetoclastic methanogenesis has a relative low upper salinity limit, while methanogens that use an

H_2/CO_2 pathway have a higher salinity tolerance - but never above 175 mg/cm^3 (Head et al., 2014).

In the experimental matrix performed here, methane production degraded with salt concentrations increasing from 3.7 mg/cm^3 to 74.3 mg/cm^3. The largest methane production occurred at the lowest salt concentration of 3.7 mg/cm. Methanogenesis was dramatically affected by large salt concentrations (>18 mg/cm^3). This detrimental effect has been also reported by Rathi et al. (2015) who used a thermophilic microbial consortium and bituminous coal. In the work of Rathi and colleagues, a decreasing trend for methane production was observed beyond 1.0 mg/cm^3. The lowest methane production was reached at 40 mg/cm^3 NaCl concentration. Maximum gas production was obtained at 1.0 mg/cm^3, close to that of the formation water from which the microbial community had been isolated.

Lavania et al. (2014) also investigated the effect of salt concentration on biologic methane production using a thermophilic consortium and subbituminous coal. High salt concentrations (70 – 100 mg/cm^3 NaCl) were detrimental. A decreasing trend of methane production was observed above 3.0 mg/cm^3 NaCl concentration. Maximum gas production occurred at a salinity at or close to the formation water (0.5 – 3.0 mg/cm^3) from which the microbial consortium had been retrieved.

Microorganisms need salts to function. These salts provide essential elements (such as sodium, potassium, and chloride) for the formation of new cells. However, salts can also act as preservatives, reducing or inhibiting microbial growth. High salt concentrations may cause the cell to expel water and lose both form and function (Liu and Boone, 1991; Schnürer and Jarvis, 2010). Low concentrations generally favor the growth of methanogens and allow greater methane production from coal (Papendick et al., 2011; Patel and Roth,

1977; Rathi et al., 2015). Salinity may control the diversity of organisms responsible for decomposition of organic matter and production of substrates necessary for methanogenesis (Chuma et al., 2016; Head et al., 2014; Waldron et al., 2007).

6.4.4.3. pH

As in all biochemical processes, pH has an important effect on methane production from complex organic matter (Del Real, 2007, Green et al., 2008). Reduced biologic activity when conditions are far from an optimum pH range can limit gas production (Del Real, 2007). Within a microbial community, methanogens are the most strongly affected by the pH (Del Real, 2007; Zupančič and Grilc, 2012). Values of pH between 6.8 – 7.4 are generally reported to be optimal for a methanogenic habitat (Del Real, 2007; Franke et al., 2014; Megonigal et al., 2013).

In this study, the experiments carried out varied pH from 4.2 to 10.2. Large methane production was obtained at a low pH value of 5.4. At pH values higher than 7.2, decreasing methane production trends were observed. Even though most methanogenic communities seem to be dominated by neutrophilic species with limited growth and methane production outside of the aforementioned optimal range (i.e., pH values 6.8 – 7.4), there are known methanogens that can exist in low pH environments (Ferry, 1993; Williams and Crawford, 1985).

Acidophilic and/or acid-tolerant strains that are able to produce methane down to pH 3.0 have been cultured (Ferry, 1993; Kotsyurbenko et al., 2007; Sizova et al., 2003; Williams and Crawford, 1985). Generally, hydrogenotrophic methanogens are more resistant to low pH than are acetoclastic methanogens (Hao et al., 2012; Kotsyurbenko et

al., 2007). Lavania et al. (2014) studied the effect of a pH range from 4.0 to 9.0 on methane production by a thermophilic consortium and subbituminous coal. Maximum production occurred at a pH of 6.5. At lower and higher pH values than 6.5, decreasing methane production trends were observed. Green et al. (2008), using a mesophilic methanogenic consortium and subbituminous coal, reported decreasing methane production when the culture medium pH was increased from 6.4 to 7.4. Volkwein et al. (1994) evaluated the methane production from coal for six different consortia on three different coals (i.e., high volatile bituminous, subbituminous and low volatile bituminous) at pH 5.0 and 7.0. Twelve of the eighteen consortium-coal combinations produced large volumes of methane at pH 5.0. In the study conducted by Zhang et al. (2016a), low pH between 6.0 and 8.0 was proven to be beneficial for bituminous coal biogasification by a mesophilic microbial consortium.

Alternatively, it has been rationalized that an acidic pH may be able to encourage methane production by enhancing coal solubility; acids may enter the coal pore structure and interact with ion-exchangeable cations, resulting in limited dissolution of coal via disruption of ionic bridges (Green et al., 2008; Lavania et al., 2014; Zhang et al., 2016a). Acids may also hydrolyze ester or ether bonds within the coal matrix (Green et al., 2008; Lavania et al., 2014).

6.4.5. Determination of a Feasible Region of Operation

The overall operational conditions for methane production are shown in Figure 20a. The methane percent concentration - Y_{CH4} was computed using the regression models (Table 21) with different values of temperature, pH, and salinity (NaCl concentration). Subsequently, these predictions were organized and visualized using a 3D scatter plot.

These predicted values were obtained up to a salt concentration of 28 mg/cm^3 where the best 3D- graphical representation was achieved. Figure 20b depicts the time- dependence for each point shown in Figure 20a. Thus, the visualization of the premium or the most representative operational conditions obtained at different periods of time was confirmed.

In Figure 20a, bright yellow describes the region that predicted the maximum methane production from subbituminous coal - Miller Black Thunder and the microbial consortium – RTTM. This region comprised salt concentrations between 3.7 mg/cm^3 and 9.0 mg/cm^3 (Figure 20a), pH values between 4.2 and 6.8, and temperatures between 23°C and 37°C, as shown in the bottom view at [NaCl] 3.7 mg/cm^3 (Figure 20c).

As other researchers (Green et al., 2008; Gupta and Gupta, 2014; Lavania et al., 2014; Rathi et al., 2015) have reported, the favorable region of operation included environmental conditions representative of the domain from where the original microbial community had been retrieved. Moderate temperature (23°C), low pH values (< 6.8), and low salt concentration (6.5 mg/cm^3 NaCl) correspond to the operational conditions imposed during microbial consortium development (Fuertez et al., 2017).

As anticipated, there is strong dependency on the environment conditions and methane production from coal. Methanogenic activity can be influenced by culturing conditions, coal bioavailability, and selectivity of the in situ coal seam environment (Fallgren et al. 2013; Rathi et al. 2015). Within the predicted region of operation, a maximum methane production is expected. In fact, these environmental conditions should favor the metabolism of the single archaea *Methanobacterium sp.*, contained in the consortium of this experimental work. The genus *Methanobacterium* has been considered to play an important role in biogenic methane production in Powder River Basin – WY,

USA (Flores et al., 2008). Additionally, this genus has been identified in the CBM reserves in Southern Qinshui Basin, China (Guo et al., 2014).

Some hydrogenotrophic methanogens that belong to the genus *Methanobacterium* have been found in moderately acidic environments. These at least transiently have excess carbon, and low or limited supply of essential minerals (i.e., some peat bogs, paddies, polluted aquifers, groundwater, and oil reservoirs). These methanogens have been shown to grow at pH 7.0 down to values as low as 3.8 and to produce some methane down to pH 3.0 (Ferry, 1993; Kotsyurbenko et al., 2007; Sizova et al., 2003; Williams and Crawford, 1985). In addition, maximum rates of methane production for pH between 5.0 and 6.0 and temperatures between 25°C and 30°C have been reported for the same genus (Kotsyurbenko et al., 2007; Sizova et al., 2003). It is known that some strains may readily adapt to extreme pH conditions. There are also alkaliphilic species (e.g., *Methanobacterium alcaliphilum*) that are able to grow at pH values up to 9.0 (Kotsyurbenko et al., 2007). As for salinity tolerance, some members of the *Methanobacterium* genus have been shown to grow and produce methane at NaCl concentrations between 2.3×10^{-2} mg/cm^3 and 15.4 mg/cm^3; other species, however, seem to be very susceptible (e.g., *Methanobacterium thermoautotrophicum*) to large salt concentrations (Patel and Roth, 1977).

6.4.6. Validation Experiment

Validation of predicted methane production for the microbial consortium, RTTM, used 6 g of subbituminous coal, tryptic soy broth (33.6% v/v, or 5.0 mg/cm^3), a salinity of 3.7 mg/cm^3 (0.37% w/v) NaCl, and was carried out at a temperature of 30°C and pH 5.5.

The maximum headspace methane was 145,165 ppm (25.6 sft^3/ton) after one-hundred and five days of incubation (Figure 21). This was considerably larger than the maximum value (76,000 ppm) measured in the earlier experimentation. For demonstration purposes, gas measurements were only conducted up to one-hundred and twelve days.

As indicated in Figure 21, there was a remarkable increase in methane production after the sixty-sixth day. This trend was in agreement with model's predictions: the largest methane concentrations would be obtained after sixty days of incubation. However, a prolonged, restricted growth phase, is observed initially. It is likely that the low initial cell concentration of 2.5 x 10^6 cfu/mL, or changes of microbial communities, and/or a transition between nutrient utilization and coal utilization at the tested conditions may had been responsible for this behavior (Green et al., 2008).

These results confirmed the suitability of the microbial consortium to produce large amounts of methane within the predicted region of operation with low concentration of nutrient amendments (33.6% v/v, 5.0 mg/cm^3 TSB). Nutrient solutions should be added at low enough volumes to maintain commercial viability (Bao et al., 2016; Zhang et al., 2016b). Favorable operational conditions were successfully recreated to maximize methane production for the microbial consortium and the subbituminous Miller Black Thunder coal. The literature reports a wide range of methane yields for different coal ranks and microbial communities exposed to various strictly anaerobic enrichment and cultivation conditions (Green et al., 2008; Gupta and Gupta, 2014; Harris et al., 2008; Jones et al., 2010; Orem et al., 2010; Papendick et al., 2011; Park and Liang, 2016; Wawrik et al., 2012). However, only a limited number of evaluations have been published where initial aerobic conditions were considered. Opara et al. (2012), for instance, reported

methane yields up to 21 sft^3/ton with addition of 50% (38.5% v/v total liquid) nutrient solution, using aerotolerant microbial consortia and bituminous Deer Creek Mine Waste coal (< 75 µm particle size, -200 mesh) at 23°C and thirty days of incubation. Similar methane yields were obtained for lignite (11 sft^3/ton) and coal waste (12 sft^3/ton) materials (Zhang et al., 2016b). Results of this study fell within this range.

6.5. Implications for Microbially Enhanced Coalbed Methane

The present work brackets environmental conditions for increased methane production from a specific subbituminous coal using a methanogenic consortium. This is the first step that one could take for upscaling to larger-scale feasibility studies. A central composite design (CCD) was used to efficiently explore a broad range of operational conditions. The experimental program assessed the effects of three environmental factors; temperature, pH, and salt concentration. An appropriate region of operation (ranges of these three environmental parameters) was identified where methane production from this coal can be maximized. An upper limit of salt concentration for viable methane production was identified. The feasible region of operation for this microbial consortium and this coal is for pH values between 4.2 and 6.8, temperatures between 23°C and 37°C, and salt concentrations between 3.7 mg/cm^3 and 9.0 mg/cm^3. Experimental verification at pH 5.5, 30°C, and a NaCl concentration of 3.7 mg/cm^3 confirmed that large methane production results within this predicted region.

Optimum laboratory conditions may not be exactly analogous to conditions found in in situ, where there is a lack of nutrients or an abundance of lack of trace elements, high in situ reservoir water pressure, plenty moisture, for instance. However, there are

documented cases for successful applications of laboratory based research in the field (Ritter et al., 2015). Some methods have been reported for laboratory determination of optimal reservoir conditions for methane production by an indigenous microbial consortium (Gupta and Gupta, 2014; Rathi et al., 2015).

Continuing on this theme, different coalbed methane reservoirs are characterized by different environments and different native microbial species. These diverse indigenous microbial populations, their function and metabolic pathways can be different from basin to basin and may change even within the same basin (Barnhart et al., 2013; Rathi et al., 2015). Other researchers have considered developing consortia specifically tailored to individual reservoirs (Gupta and Gupta, 2014; Rathi et al., 2015). This work explores the possibility of developing all-purpose, foreign, microbial consortia capable of surviving and ideally adapting to adverse environmental conditions as a low-cost biological complement.

For ex situ applications, the modification of environment conditions could readily be developed. For in situ operations, the environment is more complex and its modification may not be economically feasible, but is still achievable. For example, temperature, pH, and salinity may be changed by injecting steam, or by adding acid or other buffering agents (Green et al., 2008; Ritter et al., 2015; Zhang et al., 2016a). Adams and Opara (2015) proposed modification of in situ conditions with fluid that contains microorganisms, chemicals and/or nutrients. Ritter et al. (2015) found that for a successful in situ implementation, suitable reservoir conditions are required since the prevalent environmental conditions will affect methane generation and the resulting microbial population composition. Head et al. (2014) recognized that temperature, pH, and salinity can significantly affect methanogenesis. Study of the effects of these environmental

parameters on a selected methanogenic consortium can ensure effective in situ stimulation and/or augmentation. Lavania et al. (2014) offered similar recommendations.

Methanogenic CBM enhancement is still challenged by many uncertainties that complicate extrapolation to in situ conditions (Bao et al., 2016). However, the results generated improve our understanding of these environmental controls and provide insights for developing strategies to improve future productivity of CBM reservoirs (and/or ex situ applications of coal biogasification). The interactions among different parameters merits further study to facilitate developing an all-purpose aerotolerant system. For instance, the interaction between salinity and temperature on methanogenesis has been previously observed. Heat et al. (2014) reported less salinity tolerance at high temperatures, which may prevent biodegradation and subsequent methane production.

Among the challenges of optimizing culturing microorganisms is determining the combined effect of a multiplicity of factors that can influence their growth or the synthesis of a desired product, as well as the persistence of many complicated interactions between these factors (Fabiszewska et al., 2015). A common approach is to delineate an optimal value for one parameter, while all other conditions are maintained constant: the so-called one-variable-at-a-time technique (Green et al., 2008; Gupta and Gupta, 2014; Levania et al., 2014; Rathi et al., 2015; Zhang et al., 2016a). This practice is laborious and cost-intensive and does not allow examination of mutual interactions between factors (Fabiszewska et al., 2015). Other approaches, such as the methodology shown here, should be adopted and further examined to enable simultaneous optimization of many parameters and consequently lead to a significant reduction in the number of experiments required for quantifying a feasible range of conditions for field deployment.

Table 18. Proximate and ultimate analyses. MBT indicates Miller Black Thunder coal and RH indicates a lignite Red Hills coal

	Proximate Analysis			Ultimate Analysis	
	MBT	**RH**		**MBT**	**RH**
Moisture[a] wt.%	10.2	11.7	Hydrogen[b] wt.%	4.3	3.7
Ash[b] wt.%	6.5	24.0	Carbon[b] wt.%	67.2	51.6
Volatile[b] wt.%	48.2	43.7	Nitrogen[b] wt.%	0.9	1.1
Fixed carbon[b] wt.%	45.2	32.2	Sulfur[b] wt.%	0.3	0.8
Heating value[b] (Btu/lb)	11077	8776	Oxygen[b] wt.%	20.5	18.7
Heating value[c] (Btu/lb)	11855	11537			

[a] As received; [b] Dry basis; [c] Moisture, and ash free.

Table 19. Central composite design with results

Run #	Factors			Y_{CH4} - Experimental				
	T (ºC)	NaCl (mg/cm^3)	pH	15 days	30 days	45 days	60 days	75 days
1	39	39	7.2	0.0609	0.0582	0.0769	0.0355	0.0046
2	39	39	7.2	0.0559	0.0633	0.0664	0.0350	0.0585
3	39	39	7.2	0.0584	0.0627	0.0598	0.0344	0.0348
4	30	18	5.4	2.6442	3.1832	7.2265	21.278	0.0447
5	48	18	5.4	1.0272	10.765	5.5308	0.0521	0.0714
6	30	60	5.4	0.0520	0.0592	0.0677	0.0645	0.0664
7	48	60	5.4	0.0538	0.0626	0.0440	0.0557	0.0566
8	30	18	9.0	0.0620	0.0601	0.0309	0.0343	0.0651
9	48	18	9.0	0.0563	0.0499	0.0454	0.0421	0.0415
10	30	60	9.0	0.0531	0.0476	0.0506	0.0334	0.0033
11	48	60	9.0	0.0464	0.0522	0.0690	0.0561	0.0573
12	23	39	7.2	0.0604	0.0680	0.0743	0.0010	0.0665
13	54	39	7.2	0.0783	0.0697	0.0703	0.0612	0.0681
14	39	3.7	7.2	11.147	60.149	37.484	100	51.335
15	39	74.3	7.2	0.0554	0.0612	0.0585	0.0545	0.0600
16	39	39	4.2	0.0561	0.0562	0.0532	0.0419	0.0515
17	39	39	10.2	0.0437	0.0536	0.0559	0.0568	0.0539

The columns labeled “run,” “T,” “NaCl,” and “pH” contain the experiment run, and evaluated factors: temperature, salt concentration, and pH, respectively. The experimental design included 3 center points (runs #1-3), a factorial set 2^3 (runs #4-11), and 6 axial points (runs #12-17). Experimental results of the percent concentration – Y_{CH4} at different periods of time are shown in the right-hand side of the table.

Table 20. Analysis of variance for Y_{CH4} after sixty days

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:T	32.6018	1	32.6018	0.07	0.7986
B:NaCl	2623.47	1	2623.47	5.65	0.0491
C:pH	33.0938	1	33.0938	0.07	0.7971
AA	125.325	1	125.325	0.27	0.6193
AB	56.3472	1	56.3472	0.12	0.7378
AC	56.5237	1	56.5237	0.12	0.7374
BB	2318.97	1	2318.97	5.00	0.0605
BC	56.2985	1	56.2985	0.12	0.7379
CC	124.831	1	124.831	0.27	0.6200
Error	3248.83	7	464.119	-	-
Total	8676.29	16	-	-	-

The column labeled "Source" refers to the source of variation. It includes the effect of each factor denoted by capital letter, and the item "Error" is related to experimental error. Df refers to degrees of freedom, and it corresponds to the number of observations in the data that are free to vary when estimating statistical parameters.

Table 21. Response surface models

	Quadratic models	
t = 15 days	$Y_{CH4} = 7.01674 + 0.177371\ T - 0.490646\ NaCl + 0.427302\ pH - 0.00411721\ T^2 + 0.00106999\ T\ NaCl + 0.0123675\ T\ pH + 0.00367862\ NaCl^2 + 0.0117285\ NaCl\ pH - 0.105049\ pH^2$	$R^2 = 0.70$
t = 30 days	$Y_{CH4} = -32.1853 + 2.57839\ T - 2.0638\ NaCl + 8.7292\ pH - 0.0243579\ T^2 - 0.00500218\ T\ NaCl - 0.0585693\ T\ pH + 0.0196067\ NaCl^2 + 0.045688\ NaCl\ pH - 0.610475\ pH^2$	$R^2 = 0.67$
t = 45 days	$Y_{CH4} = 12.1331 + 0.922465\ T - 1.58028\ NaCl + 2.31875\ pH - 0.0138054\ T^2 + 0.00110846\ T\ NaCl + 0.0135204\ T\ pH + 0.0124554\ NaCl^2 + 0.0419608\ NaCl\ pH - 0.347075\ pH^2$	$R^2 = 0.71$
t = 60 days	$Y_{CH4} = 60.4177 + 1.31003\ T - 4.24967\ NaCl + 4.78854\ pH - 0.0411631\ T^2 + 0.014042\ T\ NaCl + 0.16408\ T\ pH + 0.0325223\ NaCl^2 + 0.0701797\ NaCl\ pH - 1.02705\ pH^2$	$R^2 = 0.66$
t = 75 days	$Y_{CH4} = -33.0029 + 1.92692\ T - 1.54918\ NaCl + 8.92289\ pH - 0.0247221\ T^2 + 0.0000271682\ T\ NaCl + 0.000103964\ T\ pH + 0.0160072\ NaCl^2 - 0.000174963\ NaCl\ pH - 0.619646\ pH^2$	$R^2 = 0.58$

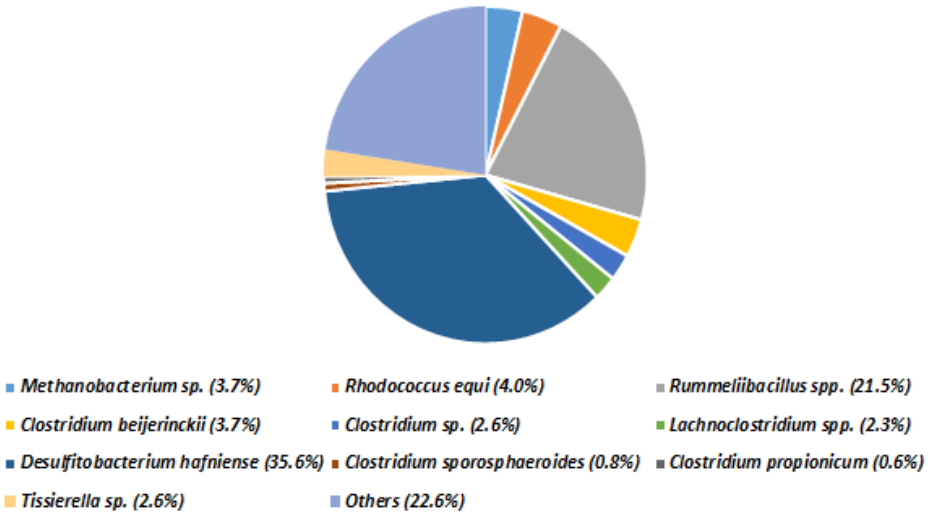


Figure 17. Diversity of microbes in the methanogenic consortium RTTM at 30°C

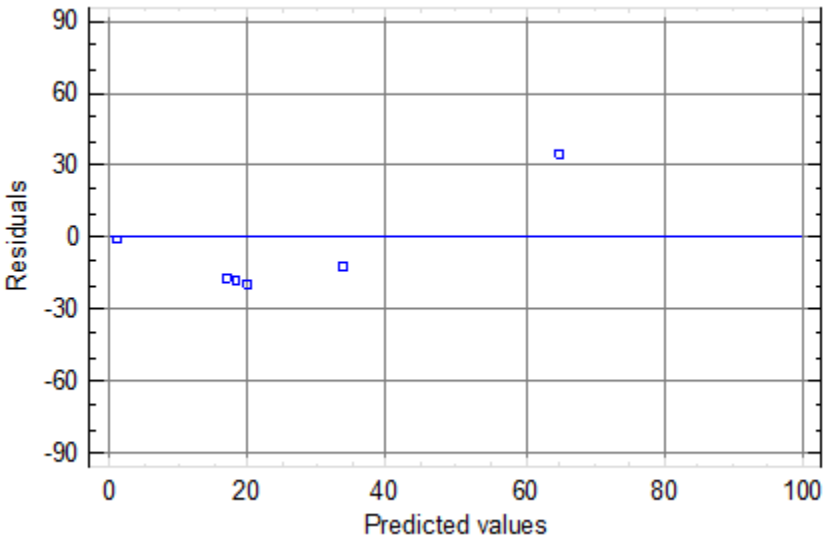
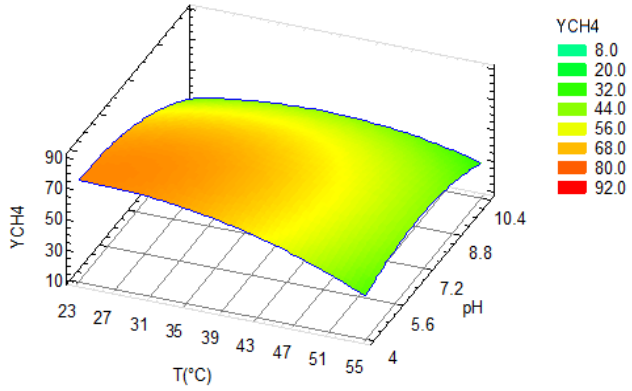
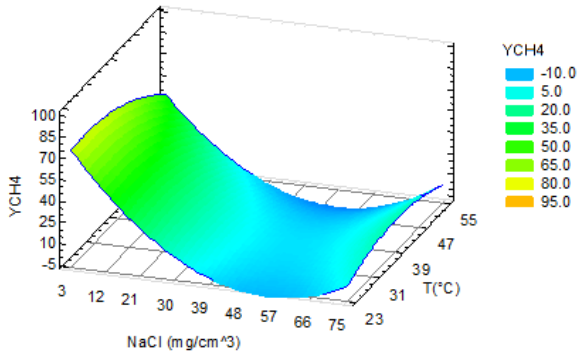


Figure 18. Plot of residuals versus predicted values of Y_{CH4}

a)



b)



c)

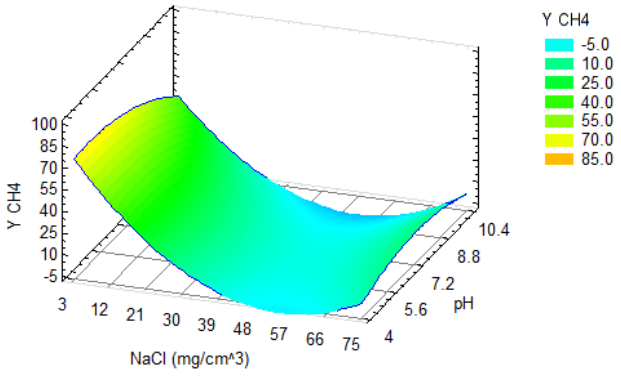
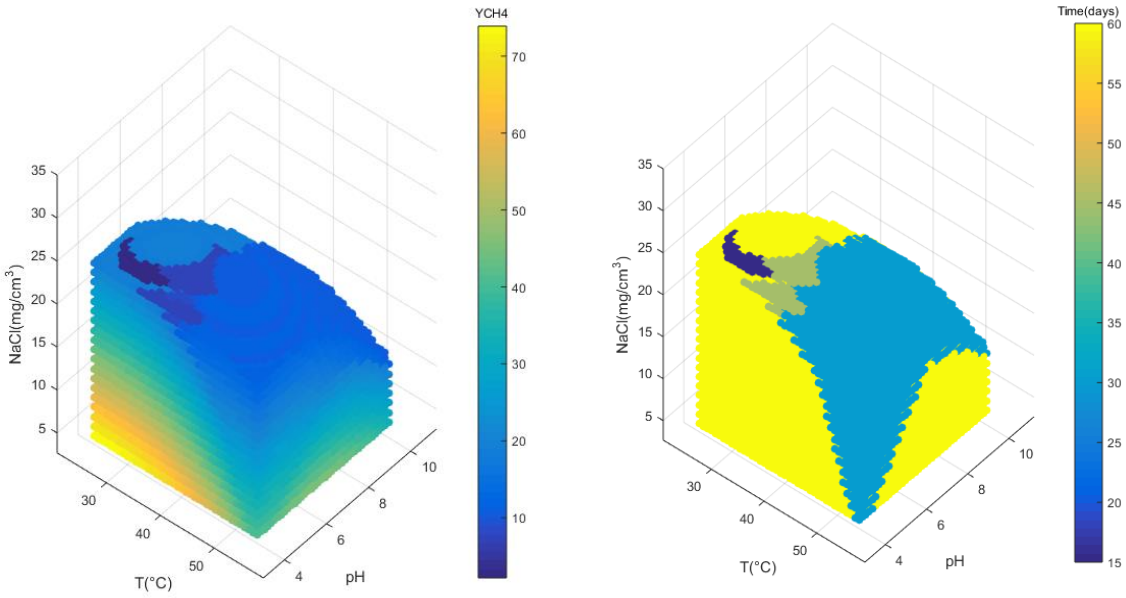


Figure 19. Response surface plots after sixty days of incubation. (a) The effect of temperature and pH on coal biogasification at [NaCl] = 3.7 mg/cm^3, (b) the effect of salt concentration and temperature on coal biogasification at pH = 5.5, (c) the effect of salt concentration and pH on coal biogasification at T = 30°C. Color bars depicts variation in methane production along the response surface.

a)

b)



c)

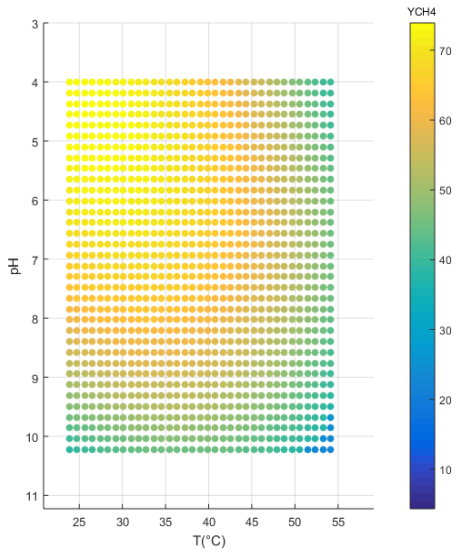


Figure 20. Overall operational conditions for methane production. (a) The effect of temperature, pH, and salt concentration on coal biogasification. Color bars depicts variation in methane production along the surfaces. (b) Dependence on time of evaluated effects on coal biogasification. Color bars depicts variation in time along the surfaces. (c) The effect of pH and temperature on coal biogasification. Bottom view at [NaCl] = 3.7 mg/cm^3. Color bars depicts variation in methane production along the surface.

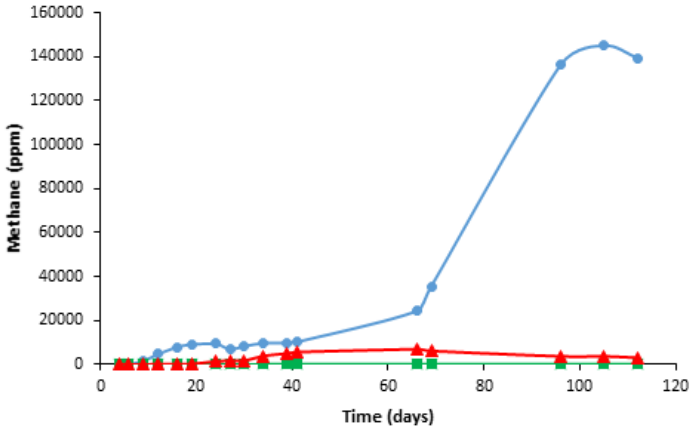


Figure 21. Verification experiment. Blue line depicts headspace methane for the microbial consortium, coal, nutrient, and salt media. The green line shows methane production for the Type I control sample (the coal and salt media). The red line is for methane content for the Type II control sample (the microbial consortium, the nutrient, and salt media). An additional control sample was used for this experiment (the coal, nutrient, and salt media), however, not significant methane production was measured (not shown in the graph).

6.6. References

Adams, D.J., Opara, A.O., 2015. Optimization of biogenic methane production from hydrocarbon sources. U.S. Patent WO 2015013531 A1.

Almeida, M., Erthal, R., Padua, E., Silveira, L., Amelia, L., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta. 76 (5), 965-977. https://doi.org/10.1016/j.talanta.2008.05.019.

ASTM E515-05, 2005. Standard Test Method for Leaks Using Bubble Emission Techniques. ASTM International, West Conshohocken, PA. doi:10.1520/E0515-05.

Bao, Y., Huang, H., He, D., Ju, Y., Qi, Y., 2016. Microbial enhancing coal-bed methane generation potential, constraints and mechanism – a mini-review. J. Nat. Gas Sci. Eng. 35, 68-78. https://doi.org/10.1016/j.jngse.2016.08.035.

Barnhart, E.P., De León, K.B., Ramsay, B.D., Cunningham, A.B., Fields, M.W., 2013. Investigation of coal-associated bacterial and archaeal populations from a diffusive microbial sampler (DMS). Int. J. Coal Geol. 115, 64-70. https://doi.org/10.1016/j.coal.2013.03.006.

Beckmann, S., Lueders, T., Krüger, M., von Netzer, F., Engelen, B., Cypionka H., 2011. Acetogens and acetoclastic methanosarcinales govern methane formation in abandoned coal mines. Appl. Environ. Microbiol. 77, 3749-3756. doi:10.1128/AEM.02818-10.

Benson, H.J., 2002. Microbiological Applications: Laboratory Manual in General Microbiology, eighth ed. McGraw Hill, New York.

Bell, K.S., Philp. J.C., Aw, D.W.J., Christofi, N., 1998. A review: The genus *Rhodococcus*. J. Appl. Microbiol. 85, 195-210. doi:10.1046/j.1365-2672.1998.00525.x.

Bergey, D.H., Holt, J.G., 1994. Bergey's Manual of Determinative Bacteriology, ninth ed. Williams and Wilkins, Baltimore.

Boone, D. R., 2015. *Methanobacterium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-8. doi:10.1002/9781118960608.gbm00495.

Botheju, D., Bakke. R., 2011. Oxygen effects in anaerobic digestion: A review. Open Waste Manag. J. 4, 1-19. doi:10.2174/1876400201104010001.

Budwill, K., 2003. Microbial methanogenesis and its role in enhancing coalbed methane recovery. CSEG Recorder. 28 (9), 41-46. http://csegrecorder.com.

Chuma, O., Elebe, N., Gideon, O., 2016. Methanogen population of an oil production skimmer pit and the effects of environmental factors and substrate availability on methanogenesis and corrosion rates. Microb. Ecol. 72 (1), 175-184. doi:10.1007/ s00248-016-0764-2.

Colosimo, F., Thomas, R., Lloyd, J.R., Taylor, K.G., Boothman, C., Smith, A.D., Lord, R., Kalin, R.M., 2016. Biogenic methane in shale gas and coal bed methane: A review of current knowledge and gaps. Int. J. Coal Geol. 165, 106-120. https://doi.org /10.1016/j.coal.2016.08.011.

Del Real, J.O., 2007. Evaluation and modeling of anaerobic depuration kinetics from stillage of alcohol industry. Ph.D. Dissertation. Institute of Basic Sciences and Engineering, Autonomous University of Hidalgo State. Mexico.

Debasmita, N., Rajasimman, M., 2013. Optimization and kinetics studies on biodegradation of atrazine using mixed microorganisms. Alexandria Eng. J. 52 (3), 499–505. https://doi.org/10.1016/j.aej.2013.06.008.

Downey, R., 2010. Method for optimizing in situ bioconversion of carbon-bearing formations. U.S. WO2010002460 A1.

Elaiyaraju, P., Partha, N., 2016. Studies on biogas production by anaerobic process using agroindustrial wastes. Res. Agr. Eng. 62 (2). 73 -82. doi:10.17221/65/2013-RAE.

Fabiszewska, A.U., Koyrba, D., Nowak, D., 2015. Assortment of carbon sources in medium for *Yarroiwia Lipolytica* lipase production: A statistical approach. Ann. Microbiol. 65 (3), 1495-1503. doi:10.1007/s13213-014-0988-7.

Fallgren, P.H., Zeng, C., Ren, Z., Lu, A., Ren, S., Jin, S., 2013. Feasibility of microbial production of new natural gas from non-gas-producing lignite. Int. J. Coal Geol. 115, 79-84. https://doi.org/10.1016/j.coal.2013.03.003.

Ferry J.G., 1993. Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman and Hall, New York.

Flores, R.M., Rice, C.A., Stricker, G.D., Warden, A., Ellis, M.S., 2008. Methanogenic pathways of coal-bed gas in the Powder River Basin, United States: The geologic factor. Int. J. Coal Geol. 76, 52-75. https://doi.org/10.1016/j.coal.2008.02.005.

Fuchs, A., Lyautey, E., Montuelle, B., Casper, P., 2016. Effects of increasing temperatures on methane concentrations and methanogenesis during experimental incubation of sediments from oligotrophic and mesotrophic lakes. J. Geophys. Res. Biogeosci. 121, 1394-1406. doi:10.1002/2016JG003328.

Fuertez, J., Boakye, R., Mclennan, J.D., Adams, D.J., Sparks, T.D., Gottschalk, A., 2017. Developing methanogenic microbial consortia from diverse coal sources and

environments. J. Nat. Gas Sci. Eng. In Press. doi:10.1016/j.jngse.2017.07.028.

Garcia, P., Brenes, M., Garrido, A., 1991. Instrumental methods for measurement of NaCl in olive brines. Fasc. 42 (4), 261-266. http://dx.doi.org/10.3989/gya.1991.v42.i4.1230.

Goodwin, S., Zeikus, J., 1987. Ecophysiological adaptations of anaerobic bacteria to low pH: Analysis of anaerobic digestion in acidic bog sediments. Appl. Environ. Microbiol. 53 (1), 57-64.

Green, M.S., Flanegan, K.C., Gilcrease, P.C., 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76, 34-45. https://doi.org/10.1016/j.coal.2008.05.001.

Guo, H., Yu, Z., Thompson, I.P., Zhang, H., 2014. A contribution of hydrogenotrophic methanogenesis to the biogenic coal bed methane reserves of Southern Qinshui Basin, China. Appl. Microbiol. Biotechnol. 98, 9083-9093. doi:10.1007/s00253-014-5908-z.

Gupta, P., Gupta, A., 2014. Biogas production from coal via anaerobic fermentation. Fuel. 118, 238-242. https://doi.org/10.1016/j.fuel.2013.10.075.

Hao, L.P., Lu, F., Li, L., Shao, L, M., He, P.J., 2012. Shift of pathways during initiation of thermophilic methanogenesis at different initial pH. Bioresour. Technol. 126, 418-424. doi:10.1016/j.biortech.2011.12.072.

Harris, S.H., Smith, R.L., Barker, Ch.E., 2008. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. Int. J. Coal Geol. 76, 46-51. https://doi.org/10.1016/j.coal.2008.05.019.

Head, I.M., Gray, N.D., Larter, S.R., 2014. Life in the slow lane: Biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. Front. Microbiol. 5, 1-23. doi:10.3389/fmicb.2014.00566.

Hoehler, T., Gunsalus, R.P., Mcinerney, J.M., 2010. Environmental constraints that limit methanogenesis, in: Timmis, K.N., McGenity, T.J., Van der Meer, J.R., De Lorenzo, V. (Eds.), Handbook of Hydrocarbon and Lipid Microbiology. Springer-Verlag Berlin Heidelberg., Berlin, pp. 635-654. doi:10.1007/978-3-540-77587-4_51.

Jones, E., Voytek, M., Corum, M., Orem W., 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76, 7013-7022. doi:10.1128/AEM.00728-10.

Khuri, A.I., Mukhopadhyay, S., 2010. Response surface methodology. WIREs Comp. Stat.

2, 128-149. doi:10.1002/wics.73.

Kiener, A., Leisinger, T., 1983. Oxygen sensitivity of methanogenic bacteria. Syst. App. Microbiol. 4 (3), 305-312. https://doi.org/10.1016/S0723-2020(83)80017-4.

Kirby, T., Lancaster, J., Fridovich, I., 1981. Isolation and characterization of the ion-containing superoxide dismutase of *Methanobacterium bryantii*. Arch. Biochem. Biophys. 210, 140-148.

Kotsyurbenko, O.R., Friedrich, M.W., Simankova, M.V., Nozhevnikova, A.N., 2007. Shift from acetoclastic to H_2-dependent methanogenesis in a West Siberian peat bog at low pH values and isolation of an acidophilic *Methanobacerium* strain. Appl. Environ. Microbiol. 73 (7), 2344-2348. doi:10.1128/AEM.02413-06.

Kreisl P., Kandler O., 1986. Chemical structure of the cell wall polymer of *Methanosarcina*. System. Appl. Microbiol. 7, 293-299. https://doi.org/10.1016 /S0723-2020(86)80022-4.

Lavania, M., Cheema, S., Manab, P., Ganapathi, R., 2014. Methanogenic potential of a thermophilic consortium enriched from coal mine. Int. Biodeterior. Biodegradation. 93, 177-185. https://doi.org/10.1016/j.ibiod.2014.05.022.

Liang, Y., 2015. Optimized microbial conversion of bituminous coal to methane for in situ and ex situ applications, in: NETL, Gasification Systems and Coal and Coal-Biomass to Liquids Workshop. Morgantown, WV. August 10-11th. https://www.netl.doe.gov/events/conference-proceedings/2015/gas-ccbtl (accessed 17.1.2017).

Liu, Y., Boone, D.R., 1991. Effects of salinity on methanogenic decomposition. Bioresour. Technol. 35 (3), 271-273. https://doi.org/10.1016/0960-8524(91)90124-3.

Megonigal, J.P., Hines, M.E., Visscher, P.T., 2013. Anaerobic metabolism: Linkages to trace gases and aerobic processes, in: Turekian, K., Holland, H. (Eds.), Treatise on Geochemistry, second ed. Elsevier Inc., Oxford, pp. 273-359. doi:10.1016/B978-0-08-095975-7.00808-1.

Meslé, M., Dromart, G., Oger, P., 2013. Microbial methanogenesis in subsurface oil and coal. Res. Microbiol. 164 (9), 959-972. https://doi.org/10.1016/j.resmic.2013.07. 004.

Myers, R.H., Montgomery, D.C., 1995. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. John Wiley and Sons Inc, New York.

Opara, A., Adams, D.J., Free, M.L., Mclennan, J., Hamilton, J., 2012. Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste

materials. Int. J. Coal Geol. 96-97, 1-8. https://doi.org/10.1016/j.coal.2012.02.010.

Opara, A., 2012. Biochemically enhanced methane production from coal. PhD. Dissertation. Department of Civil and Environmental Engineering, University of Utah. U.S.

Orem, W.H., Voytek, M.A., Jones, E.J., Lerch, H.E., Bates, A.L., Corum, M.D., 2010. Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. Org. Geochem. 41 (9), 997-1000. https://doi.org/10.1016/j.orggeochem.2010.03.005.

Papendick, S.L., Downs, K.R., Vo, K.D., Hamilton, S.K., Dawson, G.K., Golding, S.D., 2011. Biogenic methane potential for Surat Basin, Queensland coal seams. Int. J. Coal Geol. 88, 123-134. https://doi.org/10.1016/j.coal.2011.09.005.

Park, Y.S., Liang, Y., 2016. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). Fuel. 166, 258-267. https://doi.org/10.1016/j.fuel.2015.10.121.

Patel, G.B., Roth, L.A., 1977. Effect of sodium chloride on growth and methane production of methanogens. Can. J. Microbiol. 23 (7), 893-897. doi:10.1139/m77-131.

Rainey, F. A., Hollen, B. J. and Small, A. M., 2015. *Clostridium*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-122. doi:10.1002/9781118960608.gbm00619.

Rathi, R., Priya, A., Vohra, M., Lavania, M., Lal, B., Sarma, P., 2015. Development of a microbial process for methane generation from bituminous coal at thermophilic conditions. Int. J. Coal Geol. 147-148, 25-34. https://doi.org/10.1016/j.coal.2015.06.004.

Ritter, D., Vinson, D., Barnhart, E., Akob, D., Fields, M., Cunningham, A., Orem, W., McIntosh, J., 2015. Enhanced microbial coalbed methane generation: A review of research, commercial activity, and remaining challenges. Int. J. Coal Geol. 146, 28-41. https://doi.org/10.1016/j.coal.2015.04.013.

Schnürer, A., Jarvis, A., 2010. Microbiological handbook for biogas plants. Swedish Waste Management U2009:03. Swedish Gas Centre Report 207. http://www.eac-quality.net/fileadmin/eac_quality/user_documents/3_pdf/Microbiological_handbook_for_biogas_plants.pdf (accessed 15.1.2016).

Seidle, J., 2011. Fundamentals of Coalbed Methane Reservoir Engineering. Penn Well Corporation, Oklahoma.

Shah, H. N. and Hookey, J. V., 2015. *Tissierella*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New

Jersey, pp. 1-3. doi:10.1002/9781118960608.gbm00721.

Shuler, M.L., Kargi, F., 2002. Bioprocess Engineering: Basic Concepts, second ed. Prentice Hall PTR, New Jersey.

Sizova, M.V., Panikov, N.S., Tourova, T.P., Flanagan, P.W., 2003. Isolation and characterization of oligotrophic acido-tolerant methanogenic consortia from a *Sphagnum* Peat Bog. FEMS Microbiol. Ecol. 45, 301-315. doi:10.1016/S0168-6496(03)00165-X.

Sowers K.R., Gunsalus R.P., 1988. Adaptation for growth at various saline concentrations by archaebacterium *Methanosarcina thermophila*. J. Bacteriol. 170 (2), 998-1002. doi:10.1128/jb.170.2.998-1002.1988.

Stackebrandt, E., Schumann, P., 2015. *Cellulomonas*, in: Whitman, W.B. (Eds.), Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley and Sons Inc., New Jersey, pp. 1-14. doi:10.1002/9781118960608.gbm00063.

Strapoc, D., Mastalerz, M., Dawson. K., Macalady, J., Callaghan, A., Wawrik, B., Turich, C., Ashby, M., 2011. Biogeochemistry of microbial coalbed methane. Annu. Rev. Earth Planet. Sci. 39, 617-656. https://doi.org/10.1146/annurev-earth-040610-133343.

Varrone, C., Giussani, B., Izzo, G., Massini, G., Marone, A., Signorini, A., Wang, A., 2012. Statistical optimization of biohydrogen and ethanol production from crude glycerol by microbial mixed culture. Int. J. Hydrogen Energy. 37 (21), 16479-16488. https://doi.org/10.1016/j.ijhydene.2012.02.106.

Waldron, P.J., Petsch, S.T., Martini, A.M., Nusslein, K., 2007. Salinity constraints on subsurface archaeal diversity and methanogenesis in sedimentary rock rich in organic matter. Appl. Environ. Microbiol. 73 (13), 4171-4179. doi:10.1128/AEM .02810-06.

Wang, B., Tai, C., Wu, L., Chen, L., Liu, J., Hu, B., Song, D., 2017. Methane production from lignite through the combined effects of exogenous aerobic and anaerobic microflora. Int. J. Coal Geol. 173, 84-93. https://doi.org/10.1016/j.coal.2017.02 .012.

Wawrik, B., Mendivelso, M., Parisi, V.A., Suflita, J.M., Davidova, I.A., Marks, C.R., 2012. Field and laboratory studies on the bioconversion of coal to methane in the San Juan Basin. FEMS Microbiol. Ecol. 81, 26-42. doi:10.1111/j.1574-6941.2011. 01272.x.

Williams, R., Crawford R., 1985. Methanogenic bacteria, including an acid-tolerant strain from peatlands. App. Environ. Microbiol. 50, 1542-1544. PMC238796.

Wolkwein, J.C., Schoeneman, A.L., Clausen, E.G., Gaddy, J.L., Johnson, E.R., Basu, R., Ju, N., Klasson, K.T., 1994. Biological production of methane from bituminous coal. Fuel Process. Technol. 40, 339-345. https://doi.org/10.1016/03783820(94) 90156-2.

Wu, S., Bhattacharjee, A.S., Weissbrodt, D.G., Morgenroth, E., Goel, R., 2016. Effect of short term external perturbations on bacterial ecology and activities in a partial nitritation and anammox reactor. Bioresour. Technol. 219, 527-535. https://doi.org /10.1016/j.biortech.2016.07.118.

Zhang, J., Liang, Y., Pandey, R., Harpalani, S., 2015. Characterizing microbial communities dedicated for conversion of coal to methane in situ and ex situ. Int. J. Coal Geol.146, 145-154. https://doi.org/10.1016/j.coal.2015.05.001.

Zhang, J., Liang, Y., Harpalani, S., 2016a. Optimization of methane production from bituminous coal through biogasification. Appl. Energy. 183, 31-42. https://doi.org/ 10.1016/j.apenergy.2016.08.153.

Zhang, J., Park, S., Liang, Y., Harpalani, S., 2016b. Finding cost-effective nutrient solutions and evaluating environmental conditions for biogasifying bituminous coal to methane ex situ. Appl. Energy. 165, 559-568. https://doi.org/10.1016/ j.apenergy.2015.12.067.

Zupančič, G.D., Grilc, V., 2012. Anaerobic treatment and biogas production from organic waste, in: Kumar, S., Bharti, A. (Eds.), Management of Organic Waste. InTech., Rijeka, pp. 1-28. doi:10.5772/32756.

7. CONCLUSIONS AND FUTURE WORK

A methodology that involves the development of methanogenic microbial consortia, the evaluation of their potential application for coal biogasification with periodic oxygen exposure and low concentration of nutrient amendments, and the subsequent identification of a sweet pot where methane production is maximized were presented. This integrated experimental matrix can be considered as an initial step for upscaling to larger-scale feasibility studies. A summary of conclusions, significance of the results, and implications for future research and development are listed below.

- This research demonstrated that microbial communities from coal and lake sediments can be sequentially enriched and adapted through a matrix of screening/high grading steps under initial atmospheric exposure. Methanogenic consortia can be developed that could have commercial viability.
- Favorable nutrient amendments to enrich methanogenic communities were identified. This is an important element for developing microbial consortia, reducing the microbial screening requirements, and it provides possibilities for evaluating cost-effective and optimized coal biodegradation and methane production.
- The microbial consortia can be successfully reactivated and/or recovered after a long time of incubation, allowing to restimulate microbial populations and subsequently to continue the gas production from the selected coal sources.

- Significant amount of gas can be generated by methanogenic consortia under reduced concentration of nutrient amendments and periodic atmospheric exposure. This feature allows for these consortia to be considered as attractive low-cost biological complements for coal biogasification.
- The feasible coexistence of aerobes, facultative, and strict anaerobes in these consortia increases their potential to be implemented at large scale operations where oxygen exposure could exist. This includes ground bioreactors, injections into subsurface, and within biochemically degraded coal seams. Additionally, this simultaneous coexistence shown by the concurrent methane production adds to the arsenal of biodegradable capacities and potentially opens up new applications in environmental technology.
- The central composite design (CCD) was effective to explore a broad range of operational conditions, and to assess the effects of three environmental factors; temperature, pH, and salt concentration.
- An appropriate region of operation was identified where methane production from coal would be maximized. The feasible region of operation for one of the microbial consortia comprised pH values between 4.1 and 6.8, temperatures between 23°C and 37°C, and salt concentrations between 3.68 mg/cm^3 and 9.0 mg/cm^3. Experimental verification at pH 5.5, 30°C and NaCl concentration of 3.68 mg/cm^3 confirmed that large methane production is obtained in the predicted region.
- The microbial environment must be maintained within the range tolerated by the consortia. For ex situ applications, modified different parametric experimental program could readily be developed. For in situ operations, the modification of

environment is more complex and may not be economically feasible, but is still achievable.

- Methanogenic CBM enhancement is still impacted by many uncertainties that complicate extrapolation to in situ conditions. However, the results provide insights for developing strategies to improve future productivity of CBM reservoirs (and/or ex situ applications of coal biogasification).

The following items were identified as future research tasks:

- The decreasing trends in methane content that were present during experimentation, deserve further examination. Thus, effective alternatives or strategies would be proposed to maintain a stable and continuous methane production.
- The interactions among different parameters reveled by the central composite design merit further study to facilitate developing an all-purpose aerotolerant system.
- Continue to study of parameters that may affect methane production (e.g., particle size, pressure, coal loading).
- Testing of coal biogasification with continuous gas analysis for prolonged time is still needed for ex situ methane evaluation.
- An on-site pilot study for enhanced methane and carbon dioxide generation from coal-bed methane wells is still required to validate laboratory tested conditions.